

**REMARKS**

Claims 126-128, 131, 144, 149, 150, 157 and 159-164 were pending in the application at the time of issuance of the Non-Final Office Action mailed November 10, 2010. Claims 126, 128, 144 and 160-164 have been amended and new claims 165-172 have been added. Accordingly, upon entry of the amendments presented herein, claims 126-128, 131, 144, 149, 150, 157, and 159-172 will remain pending in the application.

Support for the amendments to the claims may be found throughout the specification and claims as originally filed. In particular, support for amendments to claims 126, 144, 163 and 164 may be found at, for example, page 4 lines 7-13 of the specification. Additional support for amendments to claims 126 and 144 may be found at, for example, page 8 line 14 of the specification.

Support for new claim 165 may be found at, for example, page 55 lines 16-27, page 40 lines 12-15, Figure 2A, page 43 lines 4-8, and Figures 7B and 7C of the specification.

Support for new claim 166 may be found at, for example, page 57 lines 16-27, Figure 10, and page 19 line 18 of the specification.

Support for new claims 167-171 may be found at, for example, page 6 lines 15-18 and Figure 1A.

No new matter has been added. Amendment and/or cancellation of the claims should in no way be construed as an acquiescence to any of the Examiner's rejections and was done solely in the interest of expediting prosecution and allowance of the pending claims. Applicants reserve the right to pursue the claims as originally filed in this or a separate application(s).

***Acknowledgment of Examiner's Withdrawal of Previous Rejections***

Applicants gratefully acknowledge the Examiner's withdrawal of the following: (a) the previous rejection of claim 128 under 35 U.S.C. § 112, second paragraph, (b) the previous rejection of claim 144 under 35 U.S.C. § 112, second paragraph, and (c) the previous rejection of claims 126-128, 131, 144, 149, 150, 157 and 159 under 35 U.S.C. § 112, first paragraph.

### ***Objection to the Specification***

The Examiner has maintained the objection to the specification made in paragraph 11 of the Office Action mailed 01/05/2010 with regard to claim 128. Claim 128, before the current amendment, claimed a method of resuscitating dormant moribund or latent *Mycobacterium tuberculosis* bacterial cells according to claim 126 or claim 127, wherein said bacterial cells are present in a sample, and the method identifies dormant, moribund or latent *Mycobacterium tuberculosis* bacterial cells in the sample by detecting growth of *Mycobacterium tuberculosis* bacterial cells in the sample. In the Office Action mailed 01/05/2010 the Examiner stated that there is no antecedent basis for a method of resuscitating dormant moribund or latent *Mycobacterium tuberculosis* bacterial cells according to claim 126 or claim 127, wherein the resuscitating method concurrently serves as a method of specifically identifying dormant moribund or latent *Mycobacterium tuberculosis* bacterial cells in a generic sample, or in a sample from a human or an animal.

This objection has two parts. First, the Examiner questions whether there is support for a resuscitation method that concurrently serves as a method of specifically identifying dormant, moribund or latent *Mycobacterium tuberculosis* cells. The claim has been amended to include high G+C Gram-positive bacterial cells, of which *Mycobacterium tuberculosis* cells are one example. The specification provides support for a method that serves concurrently to resuscitate and identify dormant, moribund or latent high G+C Gram-positive bacterial cells (including *Mycobacterium tuberculosis* cells). The specification explains that bacteria, including pathogenic mycobacteria such as *Mycobacterium tuberculosis*, can enter a latent or dormant state that complicates the detection, cultivation and enumeration of bacteria, for example, in the food and healthcare industries (see specification at page 2 line 22 to page 3 line 14). The specification defines RP factors as encompassing substances capable of resuscitating dormant, moribund or latent cells (e.g., bacterial cells) (page 4 lines 5-7). In addition, the specification at page 2 lines 24-28 states that resuscitation permits “non-culturable” dormant, moribund, or latent cells to become culturable. Thus, the specification explains that one of skill in the art can identify the presence of dormant, moribund or latent bacterial cells by demonstrating renewed culturability (i.e., by detecting growth following incubation in culture medium) of such dormant, moribund, or latent cells following contact of a sample containing such cells with an RP factor.

Second, the Examiner questions whether there is support for employing a generic sample,

or a sample taken from a human or animal. The specification states that the term “sample” includes samples taken from various sources, including a human or animal (see specification, e.g., at page 18 line 26) as well as soil, food, marine, freshwater, or tissue samples (see specification, e.g., at page 18 lines 25-26) and product samples, such as, for example, samples of a foodstuff, pharmaceutical preparation, or medical product (see specification, e.g., at page 31 lines 15-19). Thus, there is support in the specification for employing a sample derived from a human or animal.

In view of the foregoing, Applicants respectfully request reconsideration and withdrawal of this objection to the specification.

***Rejection of Claim 128 Under 35 U.S.C. § 112, Second Paragraph***

The Examiner has maintained the rejection made in paragraph 29(a) of the Office Action mailed 01/05/10, wherein the Examiner had rejected claim 128 as allegedly being indefinite under 35 U.S.C. § 112, second paragraph. The Examiner stated that

[i]t is unclear how a method of resuscitating a dormant, moribund or latent Mycobacterium tuberculosis cells comprising contacting the dormant, moribund or latent Mycobacterium tuberculosis cells present in a sample in vitro and incubating the cells in culture medium containing the polypeptide ends up being a method of identifying specifically “a dormant, moribund or latent Mycobacterium tuberculosis cell in the sample.”

Solely in the interest of expediting prosecution and in no way acquiescing to the validity of the Examiner’s rejection, claim 128 has been amended to state that the method identifies “the presence of dormant, moribund or latent high G+C Gram-positive bacterial cells in the sample by detecting growth of high G+C Gram-positive bacterial cells in the sample.” This amendment renders the rejection moot and Applicants respectfully request that the rejection be reconsidered and withdrawn.

Notwithstanding the foregoing, Applicants wish to make the following remarks of record. Applicants respectfully traverse this rejection for at least the following reasons. Applicants respectfully submit that, based on the teachings in the specification as well as the knowledge generally available in the art at the time the application was filed, a skilled artisan would find it clear that a method of resuscitating dormant, moribund or latent bacterial cells comprising contacting the dormant, moribund or latent bacterial cells present in a sample in vitro with the

polypeptide of claim 126 or 127 and incubating the cells in culture medium containing the polypeptide of claim 126 or 127, would provide a method of identifying the presence of dormant, moribund or latent bacterial cells in the sample. The specification provides descriptive support for a method that serves to concurrently resuscitate and identify dormant, moribund or latent bacterial cells. For example, the specification at page 2 lines 24-28 states that resuscitation permits “non-culturable” dormant, moribund, or latent cells to become culturable. Thus, one of skill in the art can identify the presence of dormant, moribund or latent bacterial cells by demonstrating renewed culturability of these cells following contact of a sample containing such cells with an RP factor, such as the polypeptide of claim 126 or 127. In view of the foregoing teachings in Applicants’ specification and the clear language of the claims, one of skill in the art would find claim 128 to be clear and definite. Accordingly, Applicants respectfully request that the Examiner reconsider and withdraw this rejection.

***Rejection of Claim 159 Under 35 U.S.C. § 112, Second Paragraph***

The Examiner has maintained the rejection of claim 159 made in paragraph 29(c) of the Office Action mailed 01/05/10 under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite because it depends from claim 128 (note that this rejection was actually made in paragraph 29(d)). The Examiner’s indefiniteness rejection of claim 128 has been addressed above. Therefore, Applicants respectfully request that the Examiner reconsider and withdraw the rejection of claim 159 under 35 U.S.C. § 112, second paragraph.

***Rejection of Claims 126-128, 131, 144, 149, 150, 157 and 159***

***Under 35 U.S.C. § 112, First Paragraph (Written Description)***

The Examiner has rejected claims 126-128, 131, 144, 149, 150, 157 and 159 under 35 U.S.C. § 112, first paragraph, as allegedly containing inadequate written description. The Examiner stated that

“[t]he description of a single species having the required function within the recited broad genus is not sufficient to support the patentability of the genus under 35 U.S.C. § 112, first paragraph. See *University of California v. Eli Lilly & Co.*, 199 F.3d 1559, 1567, 43 USPQ2d 1398, 1405 (Fed. Cir. 1997). The instant specification does not disclose which 5% of amino acid residues should be changed within the single disclosed polypeptide species of SEQ ID NO: 2 in order to maintain the required biological functions, i.e., the functional capacity to

resuscitate dormant, moribund or latent *M. tuberculosis* cells *in vitro* in a culture medium or a human or animal sample, upon performing the recited steps and/or having the ability to identify a dormant, moribund or latent *M. tuberculosis* cell in the sample by detecting growth of bacterial cells in the sample.”

Solely in the interest of expediting prosecution and in no way acquiescing to the Examiner’s rejection, claim 126 has been amended to be directed to a method of stimulating growth of high G+C Gram-positive bacterial cells or of resuscitating dormant, moribund or latent high G+C Gram-positive bacterial cells, the method comprising (i) contacting high G+C Gram-positive bacterial cells or dormant, moribund or latent high G+C Gram-positive bacterial cells *in vitro* with an isolated polypeptide having at least 50% sequence identity with amino acid residues 117 to 184 of SEQ ID NO:2, wherein said polypeptide is capable of stimulating growth of high G+C Gram-positive bacterial cells or resuscitating a dormant, moribund, or latent high G+C Gram-positive bacterial cells; and (ii) incubating said high G+C Gram-positive bacterial cells or said dormant, moribund or latent high G+C Gram-positive bacterial cells in culture medium containing the polypeptide, thereby stimulating growth of said high G+C Gram-positive bacterial cells or resuscitating said dormant, moribund or latent high G+C Gram-positive bacterial cells. Similar amendments have been made to the other independent claims.

Applicants respectfully traverse this rejection on the grounds that the instant specification sufficiently describes the claimed invention such that a skilled artisan would recognize that Applicants were in possession of the claimed invention at the time of filing. An objective standard for determining compliance with the written description requirement under 35 U.S.C. § 112, first paragraph, is whether the specification conveys with reasonable clarity to those skilled in the art that, as of the filing date sought, the applicants were in possession of the invention as now claimed. *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 1563-64, 19 USPQ2d 1111, 1117 (Fed. Cir. 1991) and *In re Gosteli*, 872 F.2d 1008, 1012, 10 USPQ2d 1615, 1618 (Fed. Cir. 1989). In the present case, the specification discloses ***at least ten species falling within the claimed genus***, i.e., ten amino acid sequences that have at least 50% sequence identity with amino acid residues 117 to 184 of SEQ ID NO:2 (including, e.g., SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:36, and SEQ ID NO:43), and ***working examples demonstrating that species falling within the claimed genus possess the claimed resuscitating and growth stimulating function***.

Applicants demonstrated the functional capacity of an RP factor purified from the supernatants of succinate-grown *Micrococcus luteus* cells (See, e.g., page 49 lines 6-16 and Figure 3B (resuscitation shown by MPN assay); Figure 4A (concentration-dependence of resuscitation shown by MPN assay); Figure 4B(stimulation of growth shown by plating); Figures 3D, 6B and Table 1 (reduction of apparent lag phase); and page 52 lines 9-24). Applicants obtained microsequences of this RP factor, from which they designed PCR primers that were used to amplify a 147 bp fragment of *M. luteus* DNA that was cloned and sequenced (see, e.g., page 49 line 25 to page 50 line 6 of the specification). Using this fragment a BLAST search was undertaken and the predicted amino acid sequence of the RP factor-encoding gene was determined (see the specification, e.g., at page 50 lines 10-11, Figure 2A and page 40 lines 11-15). The N-terminal residues of the predicted sequence (SEQ ID NO:36) agreed with the protein microsequence data, including residues not used in primer design (See, e.g., Figure 2A and page 50 lines 1-2 of the specification).

In addition, Applicants created a recombinant version of *M. luteus* RP factor, which corresponds to the secreted form of RP-factor, the sequence of which is given in Figure 2A as SEQ ID NO:43, which is identical to the portion of SEQ ID NO:36 from A39 onward (see, e.g., the specification at page 55 lines 16-17 and page 40 lines 12-15). The recombinant secreted protein also showed the expected functional capacity (see, e.g., the specification at page 55 lines 21-27, Figure 7B, and Figure 7C).

Further, Applicants created a recombinant protein based on a *Mycobacterium tuberculosis* RP factor identified in the BLAST search. This protein was the secreted version of *M. tuberculosis* RP factor, which corresponds to GI: 1655671 starting at residue D50, as stated in the specification at page 57 lines 18-19. The sequence of GI: 1655671 is disclosed in the specification as SEQ ID NO:7. The secreted version of the protein encoded by SEQ ID NO:7 also demonstrated functionality (see, e.g., the specification at page 57 lines 23-27 and Figure 10).

Applicants would like to note that in the Supplemental Amendment after Final Action dated 07-13-2010, the previous attorney of record stated that the recombinant *M. tuberculosis* RP factor was a fragment of SEQ ID NO:2 and included amino acids 117-184 of SEQ ID NO:2. In fact, as explained above, this RP factor was a fragment of SEQ ID NO:7, which showed similarity with amino acids 117-184 of SEQ ID NO:2 but did not comprise exactly this sequence.

In summary, Applicants demonstrated the functionality of a purified RP factor, a recombinant version of *M. luteus* RP factor, and a recombinant *M. tuberculosis* RP factor. The fact that each of these showed functionality demonstrates a predictable correlation between structure and function. Moreover, the Examiner acknowledges that Applicants have identified conserved structural features of RP factors that are likely to be functionally important, stating that “Applicants’ specification clearly provides guidance relating to those regions of the protein where sequence variations are likely to be tolerated and those conserved regions where variations in the sequence are less desirable.” The specification provides information regarding conserved amino acids and protein structures, for example, at page 51 line 8 to page 52 line 5, as well as Figure 1A and Figure 9. Thus, a structure-function correlation is present in the context of the present invention.

Where a structure-function correlation is present, as is the case here, even one functional species within a polypeptide genus can suffice to satisfy the written description requirement. See Example 11B, Claim 2, pages 39-42 of the *Written Description Training Materials*, (published March 25, 2008; <http://www.uspto.gov/web/menu/written.pdf>; hereinafter referred to as the “Guidelines”). The present case is similar to Claim 2 of Example 11 of the Guidelines, where the claim was to “an isolated nucleic acid that encodes a polypeptide with at least 85% amino acid sequence identity to SEQ ID NO:2; wherein the polypeptide has activity Y.” The Guidelines note that based on the disclosure of SEQ ID NO:2 and the knowledge in the art, one of skill in the art could identify and routinely generate all of the nucleic acids that encode a polypeptide with at least 85% sequence identity with SEQ ID NO:2. Similarly, here, based on the disclosure of amino acid residues 117 to 184 of SEQ ID NO:2 and the knowledge in the art, one of skill in the art could identify and routinely generate all of the polypeptides with at least 50% sequence identity with amino acid residues 117 to 184 of SEQ ID NO:2. Furthermore, one of skill in the art could predict which sequence variations would likely interfere with the claimed function based on the guidance in the disclosure as well as based on knowledge in the art about conservative substitution and empirical similarities between amino acid residues. Example 11 of the Guidelines at page 38 notes,

[f]or information on amino acid substitution exchange groups and empirical similarities between amino acid residues, see a standard text such as Schulz *et al.*, PRINCIPLES OF PROTEIN STRUCTURE, pp.14-16, Springer-Verlag (New York 1979).

There is a limit to how much substitution can be tolerated before the original tertiary structure is lost. Generally, tertiary structure conservation would be lost when the amino acid sequence varies by more than 50%. See, e.g., Cyrus Chothia and Arthur M. Lesk, "The relation between the divergence of sequence and structure in proteins," 5 THE EMBO JOURNAL 823-26 (1986).

Further, using techniques known in the art as well as methods described in the specification, one of skill in the art could test polypeptides for the ability to resuscitate dormant, moribund or latent bacterial cells (see, e.g., the specification, at page 45 line 16 to page 46 line 10; page 58 line 4 to page 59 line 19; and Figures 3, 4, 6, 7, 8, and 10). Thus, the written description provided shows that Applicants were in possession of the invention at the time of filing. More importantly, it should be noted that the written description provided in the present case is much stronger than the written description provided for Claim 2 in Example 11B of the Guidelines, because *Applicants have described at least ten species that fall within the claimed genus and have demonstrated that species falling within the claimed genus possess the claimed resuscitating and growth stimulating function.*

One of the factors to be used in determining the adequacy of written description is predictability of the aspect at issue. See, e.g., *Ariad Pharmaceuticals v. Eli Lilly*, 560 F.3d 1368, 1372. As further evidence of predictability, Applicants submit additional post-filing evidence which demonstrates the functional capacity of five species that fall within the claimed genus (i.e., Rpf A, Rpf B, Rpf C, Rpf D). Evidence demonstrating the functional capacity of Rpf D, which corresponds to the secreted version of SEQ ID NO:7, was also described in the specification, as discussed above. The additional evidence for functional capacity of claimed species is described in the following publications: Mukamolova, G.V. et al. *Molecular Microbiology* 46: 623-635 (2002) and Zhu et al. *Tuberculosis*, 83, 261-269 (2003), copies of which are included herein as Appendices A and B for the Examiner's convenience. Mukamolova et al. demonstrated the functional capacity of Rpf A, Rpf C, Rpf D, and Rpf E, which correspond to the secreted forms of the polypeptides SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:2 (i.e., the forms lacking signal sequences or N-terminal trans-membrane helices/anchors). Similarly, Zhu et al. demonstrated the functional capacity of the RpfB, which corresponds to the secreted version of SEQ ID NO:1. These five species are disclosed in the specification, for example, at page 6 lines 15-18 and Figure 1A.



In view of the foregoing, it is evident that the Applicants were in possession of the claimed invention at the time of filing. Accordingly, Applicants respectfully request that the Examiner reconsider and withdraw this rejection.

***Rejection of Claims 128, 144, 159, and 162-164***  
***Under 35 U.S.C. § 112, First Paragraph (New Matter)***

The Examiner has rejected claims 128, 144, 159, and 162-164 under 35 U.S.C. § 112 as allegedly containing new matter.

First, the Examiner requests that Applicants point to specific parts of the as-filed specification that support a method of resuscitating dormant, moribund or latent *Mycobacterium tuberculosis* cells comprising contacting the dormant, moribund or latent *Mycobacterium tuberculosis* cells present in a sample in vitro and incubating the cells in culture medium containing the polypeptide that also serves as a method of identifying specifically ‘a dormant, moribund or latent *Mycobacterium tuberculosis* bacterial cell in the sample by detecting the growth of bacterial cells in the sample.’ Applicants respectfully submit that the amendments to claims 128, 144, and 162-164 render this rejection moot.

Nonetheless, Applicants wish to make the following remarks of record. The specification provides support for a method that serves concurrently to resuscitate and identify dormant, moribund or latent high G+C Gram-positive bacterial cells (including *Mycobacterium tuberculosis* cells). The specification explains that bacteria, including pathogenic mycobacteria such as *Mycobacterium tuberculosis*, can enter a latent or dormant state that complicates the detection, cultivation and enumeration of bacteria, for example, in the food and healthcare industries (see specification at page 2 line 22 to page 3 line 14). The specification defines RP factors as encompassing substances capable of resuscitating dormant, moribund or latent cells (e.g., bacterial cells) (page 4 lines 5-7). In addition, the specification at page 2 lines 24-28 states that resuscitation permits “non-culturable” dormant, moribund, or latent cells to become culturable. Thus, the specification explains that one can identify the presence of dormant, moribund or latent bacterial cells by demonstrating renewed culturability (i.e., by detecting growth following incubation in culture medium) of such dormant, moribund, or latent cells following contact of a sample containing such cells with an RP factor.

Second, the Examiner states that a generic cell ‘strain’ expressing a nucleic acid encoding

the recited polypeptide has no support in the specification. Applicants respectfully submit that, contrary to the Examiner's assertions, such support is present in the specification. For example, at page 25 lines 16-19, the specification states that

[t]he invention also contemplates recombinant RP-factor. As used herein, the term "recombinant" is intended to define material which has been produced by that body of techniques collectively known as "recombinant DNA technology" (for example, using the nucleic acid, vectors and/or host cells described infra).

Further, at page 29 line 30 to page 30 line 1, the specification describes the "host cells" of the invention, stating that "[a]ny suitable host cell may be used, including prokaryotic host cells (such as *Escherichia coli*, *Streptomyces* spp. and *Bacillus subtilis*) and eukaryotic host cells." The fact that the specification contemplates that "[a]ny suitable host cell may be used" provides support for a generic cell strain.

In view of the foregoing teachings in the Applicants' specification it is clear that literal as well as implicit support exists in the specification for claims 128, 144, 159, and 162-164. Accordingly, Applicants respectfully request that the rejection be reconsidered and withdrawn.

### ***Rejections Under 35 U.S.C. § 112, Second Paragraph***

The Examiner has rejected claims 128, 144 and 162 as allegedly being indefinite under 35 U.S.C. § 112, second paragraph for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. Applicants thank the Examiner for the suggestions of how to respond made in paragraph 22 (a) - (c) of the Office Action. Applicants have amended claims 128, 144 and 162 in line with these suggestions.

The Examiner states that claim 162, as well as claims 128 and 159, are indefinite and confusing in the limitations 'the method identifies dormant, moribund or latent *Mycobacterium tuberculosis* bacterial cells in the sample by detecting growth of bacterial cells in the sample.' Solely in the interest of advancing prosecution and without acquiescing to the rejection, claim 162 has been amended to be directed to a method in which the dormant, moribund or latent high G+C Gram-positive bacterial cells are present in a sample, and the method identifies the presence of dormant, moribund or latent high G+C Gram-positive bacterial cells in the sample by detecting growth of high G+C Gram-positive bacterial cells in the sample. Similar amendments have been made to claims 128 and 159. Applicants respectfully submit that these amendments

render the rejections moot. Accordingly, Applicants respectfully request reconsideration and withdrawal of these rejections.

**CONCLUSION**

In view of the above amendment, Applicants believe the pending application is in condition for allowance. If a telephone conversation with Applicants' attorney would expedite the prosecution of the above-identified application, the Examiner is urged to call the undersigned at (617) 449-6512.

The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 50-4876, under Order No. 118160-00301.

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Respectfully submitted,

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# A family of autocrine growth factors in *Mycobacterium tuberculosis*

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## Summary

*Mycobacterium tuberculosis* and its close relative, *Mycobacterium bovis* (BCG) contain five genes whose predicted products resemble Rpf from *Micrococcus luteus*. Rpf is a secreted growth factor, active at picomolar concentrations, which is required for the growth of vegetative cells in minimal media at very low inoculum densities, as well as the resuscitation of dormant cells. We show here that the five cognate proteins from *M. tuberculosis* have very similar characteristics and properties to those of Rpf. They too stimulate bacterial growth at picomolar (and in some cases, subpicomolar) concentrations. Several lines of evidence indicate that they exert their activity from an extra-cytoplasmic location, suggesting that they are also involved in intercellular signalling. The five *M. tuberculosis* proteins show cross-species activity against *M. luteus*, *Mycobacterium smegmatis* and *M. bovis* (BCG). Actively growing cells of *M. bovis* (BCG) do not respond to these proteins, whereas bacteria exposed to a prolonged stationary phase do. Affinity-purified antibodies inhibit bacterial growth *in vitro*, suggesting that sequestration of these proteins at the cell surface might provide a means to limit or even prevent bacterial multiplication *in vivo*. The Rpf family of bacterial growth factors may therefore provide novel opportunities for preventing and controlling mycobacterial infections.

## Introduction

Intercellular communication between bacteria has been well documented over the last decade (Kaiser and Losick,

1993; Kell *et al.*, 1995; Salmond *et al.*, 1995; Fuqua and Greenberg, 1998). *N*-acyl-L-homoserine lactones are generally used for cell density-dependent signalling in Gram-negative organisms (Fuqua *et al.*, 1994; 1996). Peptides are more commonly, though not exclusively (Horinouchi and Beppu, 1994; Ohnishi *et al.*, 1999), used for intercellular signalling in Gram-positive organisms (Kleerebezem *et al.*, 1997; Lazazzera and Grossman, 1998). Examples of processes in which peptide-mediated signalling occurs include conjugation in enterococci (Clewell, 1993) and development of competence for genetic transformation and endospore formation in *Bacillus subtilis* (Kaiser and Losick, 1993; Lazazzera and Grossman, 1998).

Individual bacteria are normally considered autonomous, because their growth and multiplication does not apparently depend on the presence of any specific exogenous peptidic or proteinaceous growth factors (Kaprelyants *et al.*, 1994a; Votyakova *et al.*, 1994; Kaprelyants and Kell, 1996; Kell and Young, 2000). This conventional view has been challenged by the recent discovery of a protein called Rpf (*resuscitation-promoting factor*) that is secreted by growing cells of *Micrococcus luteus* (Mukamolova *et al.*, 1998). Rpf was required at picomolar concentrations for the resuscitation of dormant, 'non-culturable' cells of *M. luteus* and for the growth of small inocula in minimal media. Moreover, extensive washing of actively growing cells of *M. luteus* rendered their further growth dependent on exogenously added Rpf (Mukamolova *et al.*, 1998). It has recently been shown that *rpf* is an essential gene in *M. luteus* (Mukamolova *et al.*, 2002). Rpf therefore has the properties of a proteinaceous bacterial growth factor or cytokine (Callard and Gearing, 1994).

Genes resembling *M. luteus rpf* are widespread throughout the high G + C Gram-positive bacteria, which includes streptomycetes, corynebacteria and mycobacteria (Kell and Young, 2000). The DNA sequence databases currently contain more than 30 members of the *rpf* gene family and most organisms contain several representatives. For example, *Mycobacterium tuberculosis* and its close relative (Behr *et al.*, 1999) *Mycobacterium bovis* contain five *rpf*-like genes.

Tuberculosis, caused by *M. tuberculosis*, now kills more people in the world than any other single bacterial infection and globally, one in three people are believed to harbour a persistent (latent) infection (Bloom and Murray,

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1992; Dye *et al.*, 1999). The phenomenon of persistence has long been recognised (McDermott, 1958; Wayne, 1960; McCune *et al.*, 1966), but it remains poorly understood (Young and Duncan, 1995; Parrish *et al.*, 1998; Wayne and Sohaskey, 2001). It is generally agreed that the immune system plays an important role in preventing net bacterial multiplication (Flynn and Chan, 2001), but other aspects of the biology of the persisting organisms remain controversial. Some evidence suggests that they may be metabolically active (reviewed by Höner zu Bentrup and Russell, 2001) whereas there is also evidence that they have become dormant, or have lost culturability (Wayne, 1960, 1994). The presence of *rpf*-like genes in these mycobacteria raises the possibility that (a lack of) their products may be involved in controlling bacterial growth *in vivo*. Persisting organisms in the latent state may require one or more of these proteins in order to re-activate. To illuminate these suggestions, we have isolated recombinant forms of the five *rpf*-like proteins of *M. tuberculosis* and tested their activities using several different organisms.

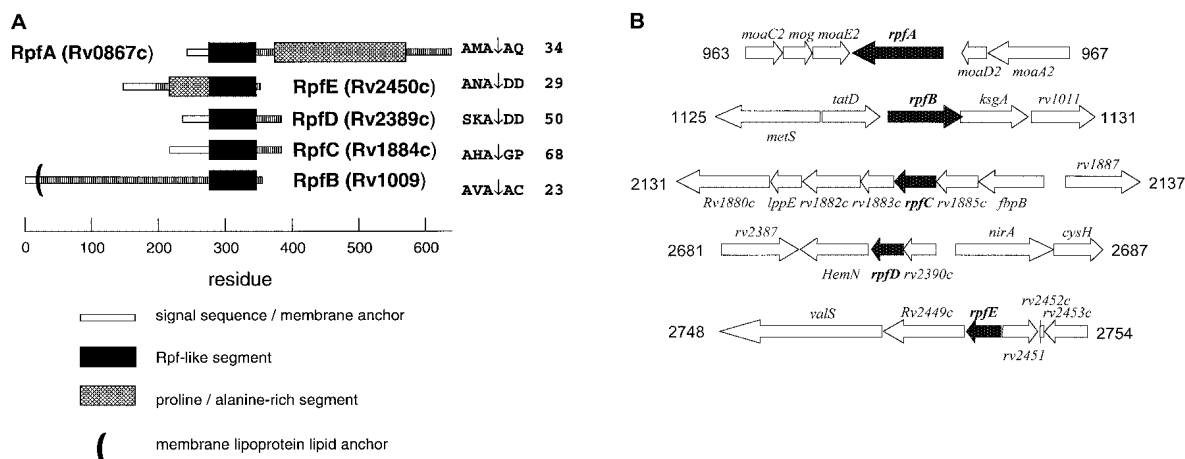
## Results

### Comparison of the five *rpf*-like genes of *M. tuberculosis*

The predicted products of the five *rpf*-like genes of *M. tuberculosis* share with Rpf a conserved ~70-residue segment (Fig. 1A). Rpf is a secreted protein. Therefore the SignalP and TMMHM servers at the Technical University of Denmark (<http://www.cbs.dtu.dk/services/SignalP/> and <http://www.cbs.dtu.dk/services/TMMHM-2.0/>) were used to determine whether the five Rpf-like proteins of *M. tuberculosis* are also likely to be secreted. Two of them,

RpfA (Rv0867c, 407 aa) and RpfD (Rv2389c, 154 aa) were predicted to be secreted proteins – see also Gomez *et al.* (2000). RpfA is a comparatively large protein in which the Rpf-like segment is followed by an extensive series (residues 146–320) of proline + alanine-rich repeats with the consensus sequence APADLAPP. The RpfB protein (Rv1009, 362 aa) has its Rpf-like domain at the C-terminus. RpfB is probably anchored to the outer surface of the cell membrane by an N-terminal prokaryotic membrane lipoprotein lipid attachment site (Prosite PS00013). Residues 1–117 of RpfB share similarity with the N-terminal Mce domain (PF02470) that is found in all six predicted products of the multiple *mce* operons of *M. tuberculosis*, at least one of which (*mce1*, Rv0169) is involved in entry into and survival inside macrophages (Arruda *et al.*, 1993). The status of the remaining two Rpf-like gene products is less clear. Although RpfC (Rv1884c, 176 aa) is not predicted to contain a *trans*-membrane helix near its N-terminus, a secretory signal sequence was predicted using a neural network (<http://www.cbs.dtu.dk/services/SignalP/>) trained on Gram-positive signal sequences. RpfE (Rv2450c, 172 aa) has a weakly predicted *trans*-membrane helix close to its N-terminus, whereas the presence of a signal sequence was quite strongly predicted. Thus, some of these five proteins are probably secreted, whereas others may be anchored in the cytoplasmic membrane. In common with Rpf, they all probably have extra-cytoplasmic functions.

The various *rpf*-like genes (Fig. 1B), are scattered about the *M. tuberculosis* genome (Cole *et al.*, 1998). The *rpfA* and *rpfE* genes appear to comprise monocistronic operons, the former lying within a cluster of genes concerned with molybdopteroate biosynthesis. There is a



**Fig. 1.** A. Schematic alignment of the five Rpf-like gene products of *M. tuberculosis*. The predicted (<http://www.cbs.dtu.dk/services/SignalP/>) signal sequence cleavage sites and the numbers of the first predicted residues of the mature secreted proteins are also indicated. B. The chromosomal context of each gene (<http://genolist.pasteur.fr/TubercuList/>). The approximate co-ordinates (kbp) of each segment are also given.

25 bp overlap between the 3' end of *rpfB* and *ksgA*, which is predicted to encode a dimethyadenosine transferase. The *rpfD* gene lies downstream from a gene of unknown function in what may be a bicistronic operon. It is located between *hemN* and *nirA*, which probably encode proteins involved in coproporphyrinogen III decarboxylation and nitrate reduction respectively. Finally, *rpfC* is the third gene in a seven-gene operon containing a mycolyltransferase (*fbpB*) upstream and a probable dehydrogenase (Rv 1882c), lipoprotein (Rv 1881c) and cytochrome P450 (Rv 1880c) downstream. The widely differing contexts of the five genes provide no clear evidence for a common biological function.

#### Biological activities of the recombinant proteins

To investigate their biological functions, the five Rpf-like proteins encoded by the *M. tuberculosis* genome were obtained in reagent quantities as polyhistidine-tagged derivatives, lacking their predicted signal sequences/membrane anchors (see Fig. 1A), as indicated in the *Experimental procedures*. They were assayed by incorporating them into the growth medium of *M. luteus* and *M. smegmatis*. We have previously shown that when these fast growing organisms are inoculated at low cell density into a minimal medium, their apparent lag phase (time to detectable turbidity) is reduced in response to Rpf addition (Mukamolova *et al.*, 1998). All four *M. tuberculosis* proteins tested also reduced the apparent lag phase of *M. luteus* (Fig. 2A). RpfA and RpfC were the most potent. The former showed activity at fM concentrations, whereas the latter caused the greatest reduction in apparent lag phase (from 216 h to 80 h). Similarly, all four proteins were active in reducing the apparent lag phase of *M. smegmatis* (Fig. 2B). At optimally active concentrations, all four proteins reduced the apparent lag phase to the same extent and for RpfA, RpfC and RpfE, maximal activity was observed at subpicomolar concentrations (Fig. 2B).

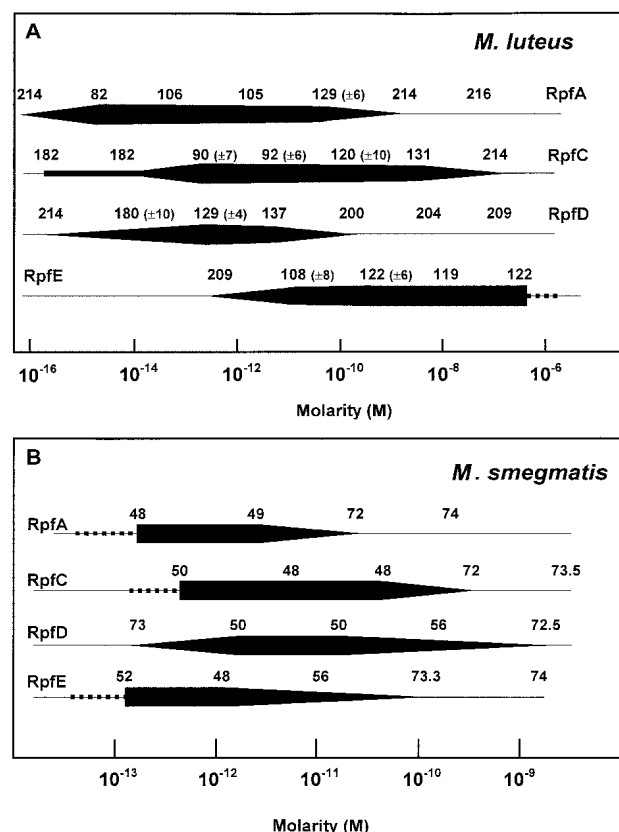
Complete activity profiles were obtained for RpfD against both organisms and for RpfA and RpfC against *M. luteus*. These profiles indicate that there is an optimal concentration range for activity, above and below which there is reduced activity or no activity at all. Indeed, the only protein for which there is no evidence of reduced activity at elevated ( $\mu$ M) concentrations, was RpfE, when tested using *M. luteus*.

The four proteins show different potency profiles when tested using these two fast-growing organisms. RpfA was active at subpicomolar concentrations against both *M. smegmatis* and *M. luteus*. On the other hand, the potency of RpfE was high when tested against *M. smegmatis*, but comparatively low when tested against *M. luteus*. Freshly purified samples were always used for experiments, because these proteins lose biological activity during stor-

age (*Experimental procedures*). Nevertheless, we cannot rule out the possibility that differences in the proportion of biologically active molecules in different protein samples could account, at least in part, for the different potencies of the four proteins. However, this cannot explain the different potency of RpfA when tested with the two different organisms, because the assays were done at the same time with the same protein preparation.

*Mycobacterium bovis* (BCG) was chosen as a representative of the slow-growing mycobacteria (Wayne, 1984) with which to test the biological activities of the Rpf-like proteins of *M. tuberculosis*. *Mycobacterium bovis* (BCG) is closely related to *M. tuberculosis* (Behr *et al.*, 1999) and contains five *rpf* homologues that are very similar indeed to those of *M. tuberculosis* (Kell and Young, 2000).

The response of *M. bovis* (BCG) to the five proteins



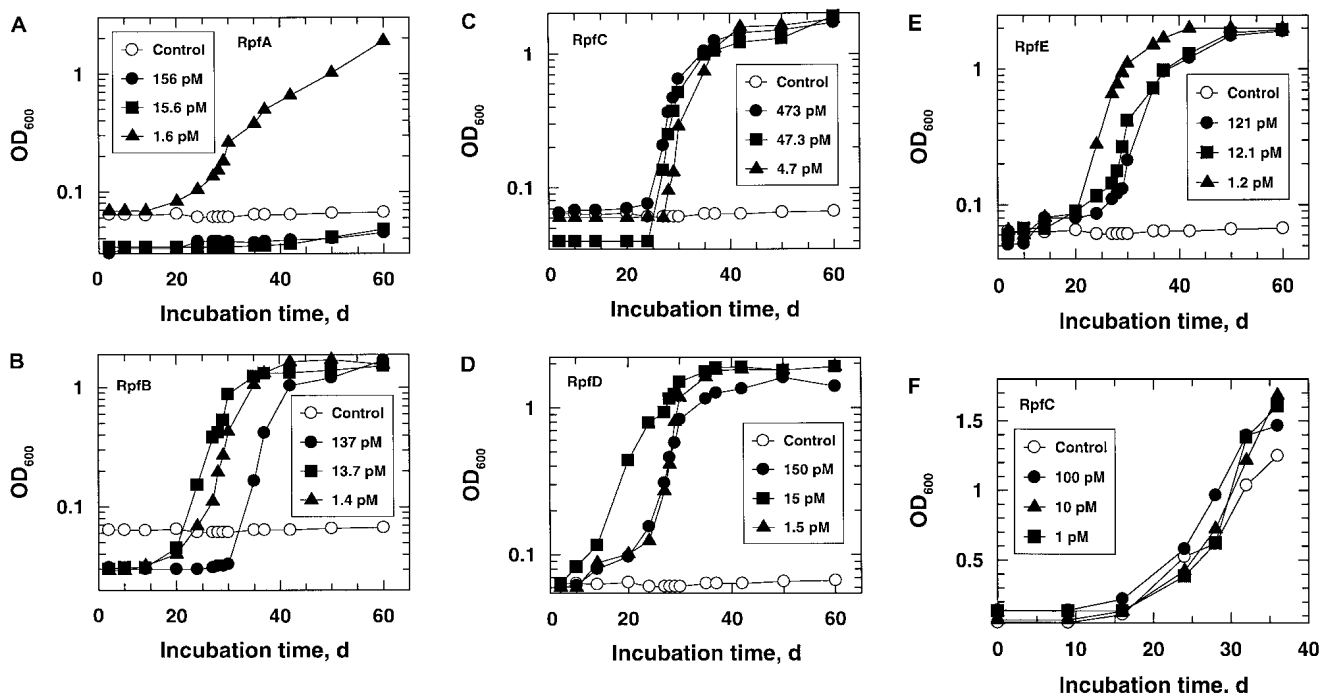
**Fig. 2.** The Rpf-like proteins of *M. tuberculosis* reduce the apparent lag phase of *M. luteus* (A) and *M. smegmatis* (B). Protein samples were serially diluted 30-fold (A) or 10-fold (B). The mean apparent lag phase values are shown at each protein concentration tested ( $\pm$ SD, if this was greater than 3 h). Activity is represented schematically by the filled shapes, whose width is maximal at protein concentrations showing maximum activity (reduction of apparent lag phase, i.e. time to measurable turbidity in the Bioscreen C growth analyser). A dotted line indicates that activity is presumed to extend beyond the lowest or highest dilution tested. In controls with no added protein, the apparent lag phase was  $216 \pm 4$  h (*M. luteus*) and  $74 \pm 4$  h (*M. smegmatis*).

depended on the age of the inoculum. When actively growing cells were used, none of the proteins stimulated bacterial growth (representative data are shown for RpfC in Fig. 3F). However, in common with cells of both the virulent and the avirulent strains of *M. tuberculosis* (Sun and Zhang, 1999; Zhang *et al.*, 2001; Shleeva *et al.*, 2002), those of *M. bovis* (BCG) lose culturability during extended stationary phase. The activities of the five Rpf-like proteins of *M. bovis* (BCG) were therefore monitored using late stationary phase cells. As indicated in Fig. 3A–E, all five proteins were active; growth usually occurred after an appreciable lag, and was dependent on the provision of pM concentrations of any one of these five proteins. RpfA only showed activity at the lowest concentration tested (1.6 pM) (Fig. 3A), whereas the other proteins were active over the entire concentration range tested. The data summarized in Fig. 2A–B and Fig. 3A–E establish that the *rpf*-like genes of *M. tuberculosis* encode a family of growth factors with activities similar to that of *M. luteus* Rpf (Mukamolova *et al.*, 1998).

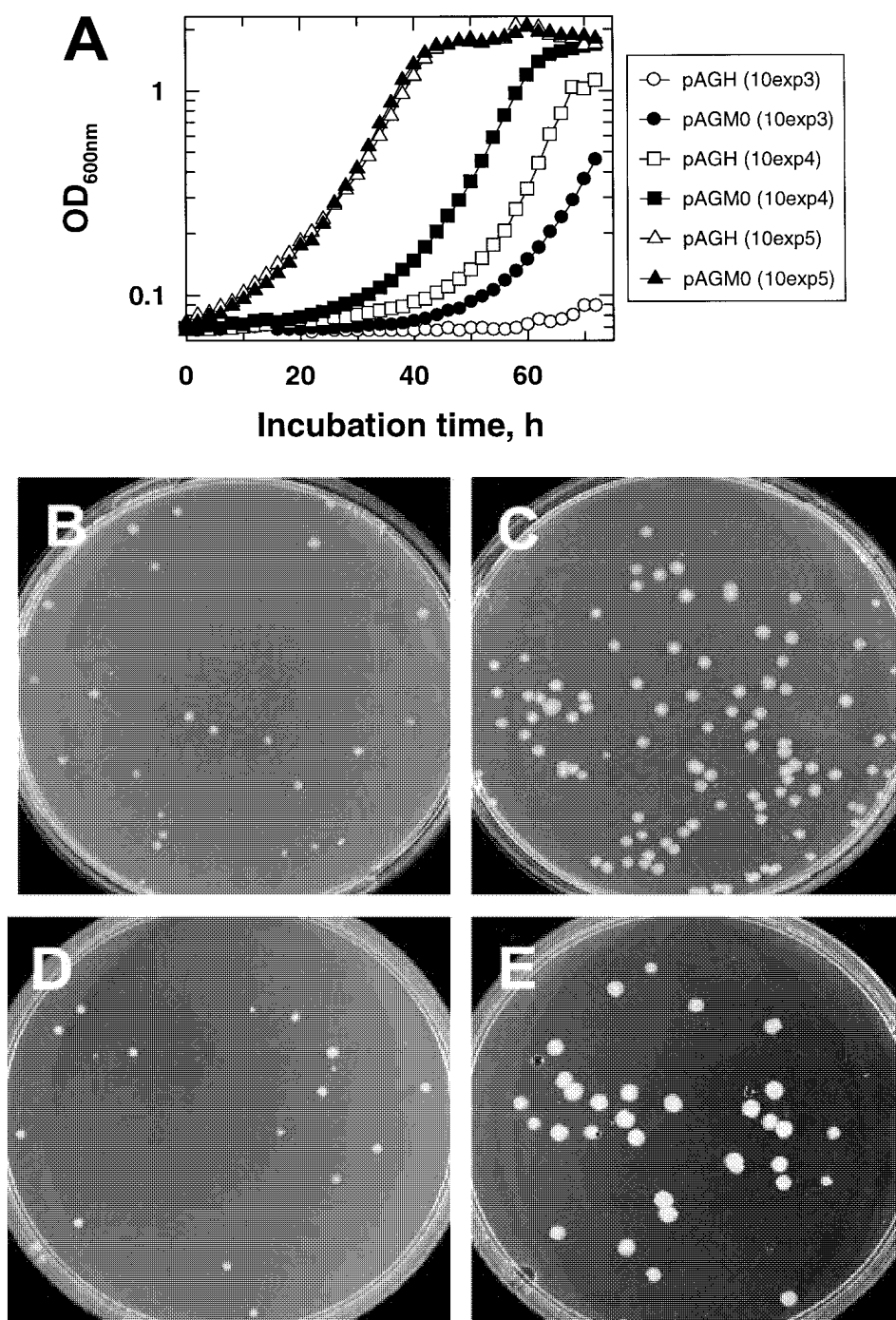
#### Rpf expression in *M. smegmatis* stimulates bacterial growth

To circumvent potential problems arising from the fact that the recombinant proteins are unstable and the proportion of biologically active molecules in different prepa-

rations of recombinant proteins is not known (see above), we examined the effect of Rpf expression *in vivo*. Plasmid pAGM0 (Mukamolova *et al.* 2002), expressing *rpf* under the control of the *M. smegmatis* amidase promoter ( $P_{ami}$ ) (Parish *et al.*, 1997) was introduced into *M. smegmatis*. Using reverse transcriptase-polymerase chain reaction (RT-PCR), we verified that *rpf* is expressed soon after inoculation of the pAGM0-containing strain into fresh growth medium (data not shown). The appearance of Rpf in the culture supernatant was also demonstrable by Western blotting of samples taken when the cultures were in exponential phase ( $OD_{600} = 0.6$ ). Using an inoculum grown to stationary phase overnight in NBE, bacteria were subjected to nutritional shift-down by inoculation at three different densities into Sauton's medium. At low inoculum densities ( $10^3$  and  $10^4$  colony-forming units (cfu)  $ml^{-1}$ ), the apparent lag phase was substantially reduced in the Rpf-expressing strain compared with that of the control containing the pAGH vector (Fig. 4A). This effect was observed in the presence of either kanamycin or hygromycin, used to select for plasmid maintenance and also in the absence of either antibiotic (data not shown). It was also observed in the absence of acetamide, consonant with the report that this promoter is partially active in the absence of inducer (Parish *et al.*, 1997). Growth stimulation was not apparent at a higher inoculum density ( $10^5$  cfu  $ml^{-1}$ ), nor was it ob-



**Fig. 3.** The Rpf-like proteins of *M. tuberculosis* stimulate the growth of *M. bovis* (BCG). The responses of aged *M. bovis* (BCG) (5 month-old culture) to recombinant versions of RpfA (A), RpfB (B), RpfC (C), RpfD (D) and RpfE (E) are shown. The response of actively growing *M. bovis* (BCG) (inoculum was 100 cells  $ml^{-1}$  from late-logarithmic phase) to recombinant RpfC (F) is also shown. The  $OD_{600}$  of each culture was monitored with time.



**Fig. 4.** Comparative growth kinetics (A) of *M. smegmatis* strains containing plasmids pAGH (vector) and pAGM0 (*rpf* expressed from  $P_{ami}$ ) using Sauton's medium containing 0.05% Tween-80 and 5 µg kanamycin ml<sup>-1</sup>. The number of cfu in the overnight cultures (adjusted to OD<sub>600</sub> = 1.0, and used as inocula for this experiment) were  $1.23 (\pm 0.11) \times 10^8$  for the control containing pAGH, and  $1.03 (\pm 0.11) \times 10^8$  for the strain containing pAGM0. The remainder of the Figure shows colonies obtained from bacteria containing pAGH (B and D) or pAGM0 (C and E) 72 h post inoculation, on Sauton's medium containing ADC and either 50 µg hygromycin ml<sup>-1</sup> (B and C) or 10 µg kanamycin ml<sup>-1</sup> (D and E).



served in a strain harbouring pAGM1, in which  $P_{ami}$  generates a counter-transcript of *rpf*. The growth-stimulatory effect of *rpf* expression in *M. smegmatis* was also evident when comparing the sizes of colonies of strains harbouring pAGM0 (expressing *rpf*) and pAGH (vector control) obtained on agar-solidified Sauton's medium, both in the presence and absence of selective antibiotics (Fig. 4B–E and data not shown).

#### Expression of the five *rpf*-like genes of *M. tuberculosis* and *M. bovis* (BCG)

Messenger RNA corresponding to all five *rpf*-like genes was detected by RT-PCR using RNA isolated from cells of *M. tuberculosis* H37Rv and *M. bovis* (BCG) growing actively *in vitro* (Fig. 5A and B). Although only weak signals were obtained for RpfD (Rv2389c) in *M. bovis* (BCG) and RpfE (Rv2450c) in both organisms, expression of both genes has been detected by others in microarray experiments (Manganelli *et al.*, 2001; Sherman *et al.*, 2001). In contrast, we were unable to detect mRNA corresponding to any of these genes in RNA extracted from *M. bovis* (BCG) during stationary phase, nor from cells that had been starved for 5 months (data not shown). All five *rpf*-like genes are expressed in actively growing cells, whereas non-growing cells express them either at levels that are below the limit of detection, or not at all.

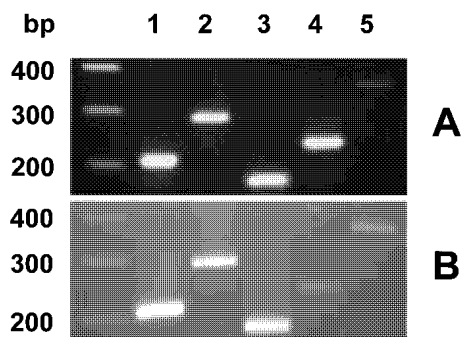
To monitor protein production, antibodies were raised against a histidine-tagged, truncated form of Rpf comprising residues A<sub>42</sub>–L<sub>118</sub>, i.e. the conserved 'Rpf domain' that is shared by all family members (Kell and Young, 2000), and purified by affinity chromatography (*Experimental procedures*). These antibodies reacted with recombinant versions of all five of the Rpf-like proteins of *M. tuberculosis* (Fig. 6C). They detected two bands in concentrated sam-

ples of supernatant obtained from exponentially growing cultures of *M. bovis* (BCG) (Fig. 6A) and *M. tuberculosis* (data not shown). Several bands were also detected in concentrated samples of supernatant obtained from exponentially growing cultures of *M. smegmatis* (Fig. 6B). Only the uppermost band was seen (and its apparent size was slightly reduced) if protease inhibitors were not present during isolation (see *Experimental procedures*). According to the available genome sequence information ([http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/genom\\_table.cgi](http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/genom_table.cgi)), this organism potentially produces four Rpf-like proteins. These experiments confirm that Rpf-like proteins are detectable in the supernatant of actively growing cultures of *M. bovis* (BCG), *M. tuberculosis* and *M. smegmatis*.

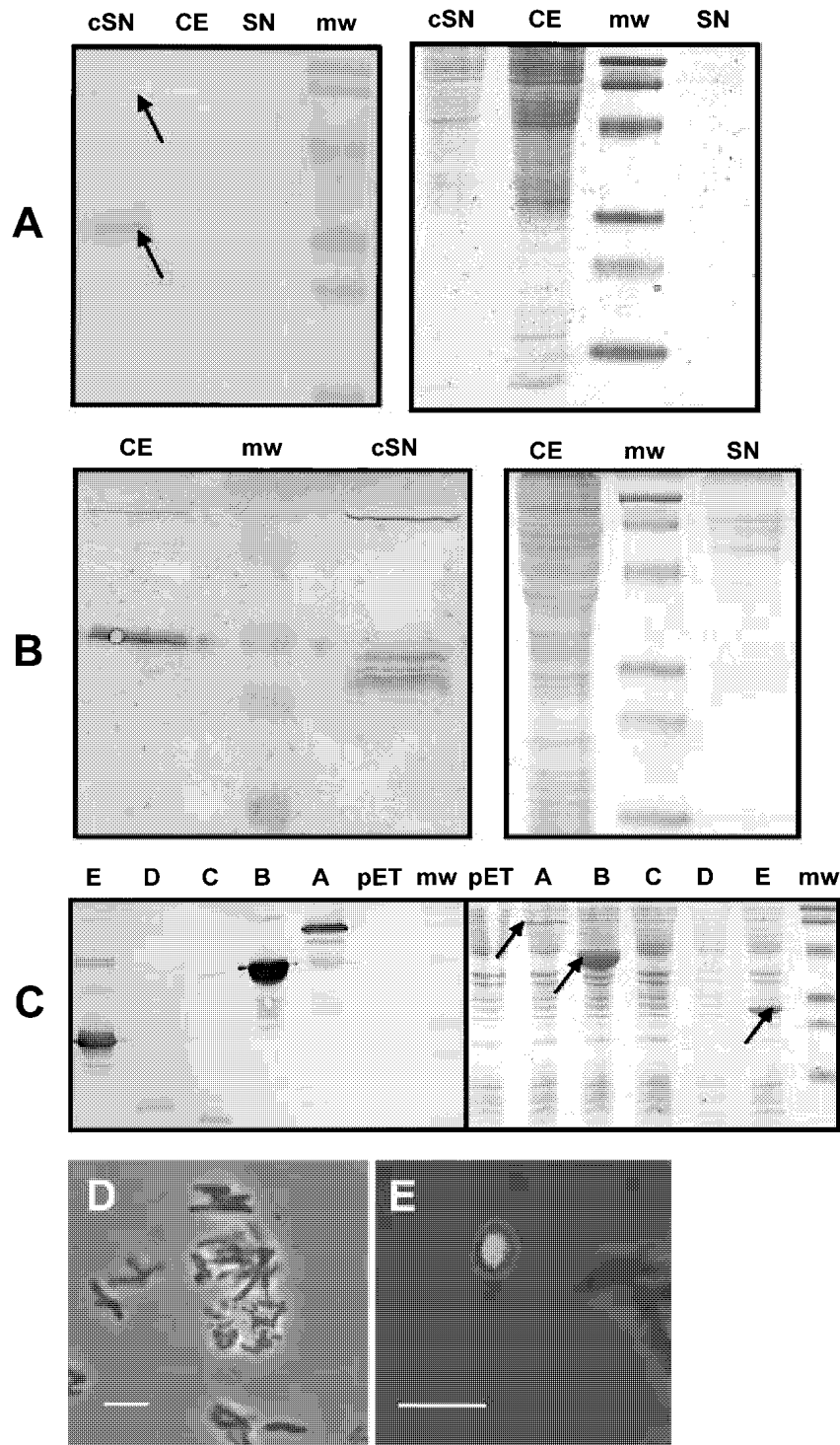
Using confocal microscopy of *M. bovis* BCG that had been incubated with anti-truncated Rpf antibodies and a secondary, FITC-labelled antibody, Rpf-like protein(s) were detected on the bacterial cell surface. About 30% of the *M. bovis* (BCG) cells in early logarithmic phase cultures (8 days post inoculation) showed detectable fluorescence and some of them were much more strongly fluorescent than others (Fig. 6D and E). Fluorescence was abolished if recombinant Rpf was added to the bacteria at the same time as the primary anti-Rpf antibody. None of the cells in stationary phase cultures (6 weeks post inoculation) showed visible fluorescence (data not shown).

#### Anti-Rpf antibodies inhibit bacterial growth

Given the accumulated evidence that members of the Rpf protein family stimulate bacterial growth from an extra-cytoplasmic location, we determined the effect of adding anti-Rpf antibodies to the culture medium. Immunoglobulins purified from immune serum partially inhibited the growth of the avirulent Academia strain of *M. tuberculosis*, whereas immunoglobulins purified from preimmune serum were without effect (Fig. 7A). Moreover, the inhibitory effect of immunoglobulins in the immune serum was overcome by the addition of Rpf. Similar growth inhibition of *M. bovis* (BCG) was observed using affinity-purified anti-Rpf antibodies. Inhibition was transient, resulting in delayed bacterial growth when using a large inoculum of late logarithmic cells (Fig. 7B). This was probably not a result of antibody degradation during the long incubation period, as anti-Rpf antibodies were detectable in the culture medium by Western blotting throughout the experiment (data not shown). There was more pronounced growth inhibition using a small inoculum of aged cells of *M. bovis* (BCG) (Fig. 7C). In all of these experiments, the addition of Rpf overcame the growth-inhibitory effect of the anti-Rpf antibodies. Indeed, in Fig. 7C the provision of Rpf abolished the short lag phase seen in the control.



**Fig. 5.** The *rpf*-like genes of *M. tuberculosis* (A) and *M. bovis* (BCG) (B) are expressed *in vivo*. RT-PCR products obtained from *M. tuberculosis* H37Rv (A) and *M. bovis* (BCG) (B). RNA, using primer pairs specific for: 1, RpfA (209 bp); 2, RpfB (288 bp); 3, RpfC (180 bp); 4, RpfD (238 bp); 5, RpfE (357 bp) – sizes of expected products in parentheses. Markers of 200, 300 and 400 bp derived from a 100 bp ladder (Promega) are also shown.



**Fig. 6.** Detection of Rpf-like proteins in culture supernatant from *M. bovis* BCG (A) and *M. smegmatis* (B) and on the surface of cells of *M. bovis* BCG (D and E). In A and B, samples of crude cell extract (CE), culture supernatant (SN) and proteins concentrated from culture supernatant using DEAE cellulose chromatography (cSN) are shown. C shows the recombinant Rpf-like proteins of *M. tuberculosis* in crude extracts of *E. coli* HMS174 following IPTG-induced expression (lanes A–E correspond to strains expressing RpfA–E; lanes labelled pET contain the pET-19b vector). In A–C, the left-hand panels show proteins detected by the rabbit anti-Rpf antibody and the right-hand panels show the corresponding Coomassie blue-stained gel. The size markers in A–C (98, 66, 45, 30, 22, 17 kDa), were from BioRad. The arrows in A indicate the positions of proteins detected on the immunoblot and those in C, indicate the positions of recombinant RpfA, B and E in the Coomassie blue-stained gel. C shows evidence of both aggregation and degradation of RpfA, RpfB and RpfE. D and E show confocal microscope images of fixed cells of *M. bovis* BCG from an exponential phase culture (8 days post inoculation) following incubation with anti-truncated Rpf antibodies and a secondary FITC-labelled antibody. E is a view of the pole of a fluorescent cell.

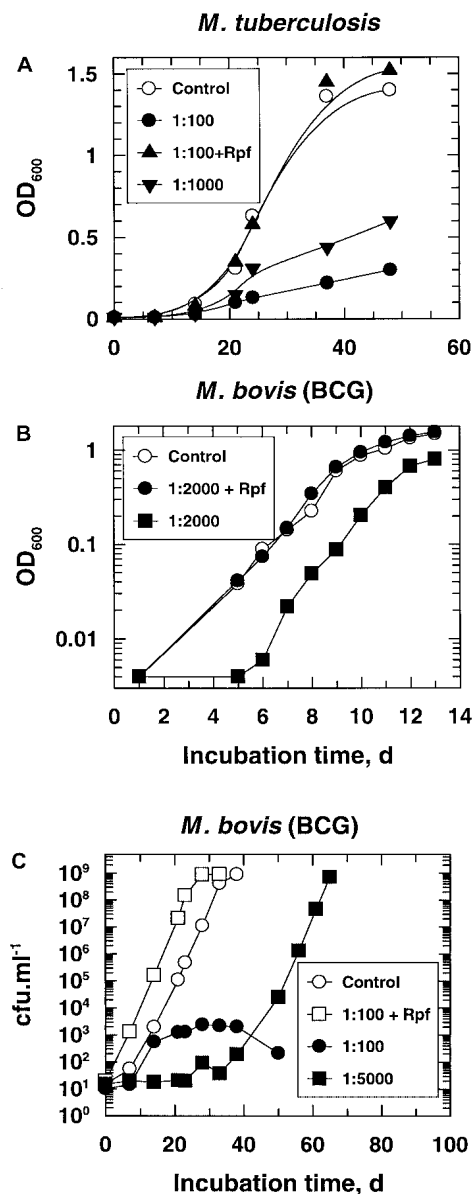
## Discussion

Information from the published genome sequence (Cole *et al.*, 1998) suggested that the *rpf*-like genes of *M. tuberculosis* encode a family of surface-located or secreted proteins. This was confirmed by the results reported here. One or more of these proteins was detected on the surface of actively growing bacteria and in concentrated samples of supernatants obtained from cultures of *M.*

*tuberculosis*, the closely related organism, *M. bovis* (BCG), and *M. smegmatis* (Fig. 6A and B). Moreover, growth of *M. bovis* BCG was stimulated by recombinant versions of these Rpf-like proteins (Figs 2 and 3), and inhibited when anti-Rpf antibodies were incorporated into the culture medium (Fig. 7).

The Rpf-like proteins of *M. tuberculosis* stimulated bacterial growth in laboratory culture at very low (pM) concentrations, which effectively excludes the possibility that they were simply being used as nutrients. The most potent proteins were active at concentrations equivalent to just a few molecules per cell (Fig. 2), consistent with the view that they act as growth factors. As was previously noted when *M. luteus* Rpf was tested against a panel of different organisms, the Rpf-like proteins of *M. tuberculosis* show cross-species activity. They stimulated the growth of the closely related, slow-growing organism, *M. bovis* (BCG) (Fig. 3) as well as that of two fast-growing organisms, *M. smegmatis* and *M. luteus* (Fig. 2). Moreover, expression of *rpf* in *M. smegmatis* also stimulated the growth of this organism in a minimal medium (Fig. 7), confirming the results obtained with exogenously added recombinant proteins. It is noteworthy that this represents the first demonstration of Rpf-mediated growth stimulation on a solid medium; when recombinant proteins are incorporated into molten agar or spread on the surface of agar plates they are not active (Kaprelyants *et al.*, 1994; Mukamolova *et al.*, 2002).

Reverse transcriptase–polymerase chain reaction showed that actively growing cells of both *M. tuberculosis* H37Rv and *M. bovis* (BCG) express all five of their *rpf*-like genes (Fig. 5) and Western blotting indicated that Rpf-like proteins are detectable in the culture medium (Fig. 6A). Continued cellular multiplication is assured by endogenous protein production; hence, the provision of extra molecules in the culture medium has little effect on bacterial growth (Fig. 3F and unpublished data). Moreover, some organisms appear to produce sufficient amounts of these growth factors to render them relatively insensitive to the growth-inhibitory effect of anti-Rpf antibodies (Fig. 7B) and to permit their detection on the cell surface (Fig. 6D–E). Non-growing bacteria do not produce these growth factors. As they remain in stationary phase, previously synthesized molecules gradually decay, and the bacteria become increasingly dependent on an exogenous supply in order to resume growth (Fig. 3A–E). A growth factor requirement is also detectable in cells of the avirulent Academia strain of *M. tuberculosis* that have persisted for several days within murine macrophages (Biketov *et al.*, 2000). Mycobacteria inoculated from late stationary phase, or even older cultures, either fail to grow, or grow only very slowly after an extended lag phase (Dubos and Davis, 1946; Aldovini *et al.*, 1993; Yuan *et al.*, 1996; Lim *et al.*, 1999; Sun and Zhang, 1999). The addi-



**Fig. 7.** Influence of anti-Rpf antibodies on bacterial growth. Cultures were inoculated with  $10^5$  cfu from a 2-month-old culture of *M. tuberculosis* (A),  $10^6$  cfu from a 2-week-old culture of *M. bovis* (BCG) (B) and  $10^2$  cfu from a 6-week-old culture of *M. bovis* BCG (C). The IgG fraction purified from immune and preimmune sera (A) and affinity-purified rabbit anti-Rpf antibodies (B and C) were used and Rpf (450 pM) was added as indicated. Growth was monitored by measuring the OD<sub>600</sub> (A and B) or by plating (C).

tion of Rpf-like growth factors to such cultures should aid the resumption of normal growth, which could potentially improve the diagnosis and quantification of mycobacterial infections.

To obtain 'non-culturable', Rpf-responsive cells of *M. bovis* (BCG) (see Fig. 3), bacteria were incubated in Sauton's medium in prolonged stationary phase, without exposure to the laboratory atmosphere. These conditions (except for oxygen depletion) were similar to those used previously to elicit dormancy in *M. luteus* (Mukamolova *et al.*, 1995). The avirulent H37Ra strain of *M. tuberculosis* can also enter a 'non-culturable' state after prolonged stationary phase, from which it can be resuscitated using culture supernatants (Sun and Zhang, 1999). Using an indirect, fluorescence-based assay that correlated with resuscitation, the authors initially reported that the active agent was a small MW, heat-stable compound that accumulated in early stationary phase. Subsequently (Zhang *et al.*, 2001), activity in early stationary phase supernatants was associated with phospholipids and an 8 kDa protein (Rv1174c). Three synthetic peptides representing different segments of this protein were active when added to bacteria at micromolar concentrations. In our experiments, in which minute (picomolar) concentrations of Rpf-like proteins permitted the growth of organisms that were otherwise non-culturable (Fig. 3A–E), we can effectively exclude any non-specific growth stimulation potentially arising from utilization of these molecules as nutrients.

A growth factor requirement can be artificially induced in growing cells of *M. luteus* by repeatedly washing them and inoculating them at low cell density (Mukamolova *et al.*, 1998; 1999). Rpf has a LysM lysin domain at its C-terminus, which is probably a peptidoglycan-binding module (Bateman and Bycroft, 2000). Washing presumably removes secreted Rpf molecules associated with the cell envelope. Attempts to induce a similar growth factor requirement in *M. tuberculosis* and *M. bovis* (BCG) by washing have proved unsuccessful (unpublished data). This is consistent with the observation that one or more of the Rpf-like proteins elaborated by these organisms, which tend to form extensive clumps, cords and pellicles, is probably membrane-bound (e.g. RpfB).

We suspect that the five different Rpf-like proteins of *M. tuberculosis* and *M. bovis* (BCG) fulfil subtly different, overlapping, biological functions. They may all act as autocrine signalling molecules, effectively stimulating the growth and multiplication of the cells that produce them. Free-living (planktonic) cells may use secreted proteins (e.g. RpfA) for paracrine, density dependent signalling (quorum sensing) (Fuqua *et al.*, 1996), whereas cells growing in close proximity may use envelope-associated proteins for juxtacrine signalling (Kaprelyants *et al.*, 1999). Dormant or injured cells (defined in Kell *et al.*, 1998) require an exogenous source of these proteins for their

resuscitation (Mukamolova *et al.*, 1998; Biketov *et al.*, 2000). Further insights into their precise biological roles will require the construction and analysis of mutants harbouring null mutations.

The growth-promoting effects of these proteins appear to result from their specific interaction with a component of the bacterial cell envelope. Two lines of evidence suggest that they bind to specific receptors. One is their extreme potency. The other is that they have an optimum concentration for biological activity, above which they are either inactive or even inhibitory (Mukamolova *et al.*, 1998; 2002). Evidence of this nature has previously been adduced in studies of hormone–receptor and ligand–receptor interactions (Franklin, 1980; Gero, 1983).

When mycobacteria experience an extended stationary phase *in vitro* they often lose culturability (Sun and Zhang, 1999; Zhang *et al.*, 2001; Shleeve *et al.*, 2002). They may possibly enter a state of Rpf-responsive dormancy, akin to that observed when *M. luteus* experiences prolonged stationary phase (Kaprelyants and Kell, 1993; Kaprelyants *et al.*, 1993). Cells that have been starved *in vitro* may be physiologically similar to bacteria that persist *in vivo*, in patients harbouring a latent *M. tuberculosis* infection (Parrish *et al.*, 1998). Persisting organisms are detectable (de Wit *et al.*, 1995; Hernandez-Pando *et al.*, 2000; Pai *et al.*, 2000), but cannot be cultured using conventional methods (plating or inoculation into liquid media) (Wayne, 1960, 1994; McCune *et al.*, 1966; de Wit *et al.*, 1995). It is consequently possible that (a lack of) Rpf-like growth factors contributes a microbiological component to the phenomenon of mycobacterial persistence in latent infections. The results reported here indicate that the Rpf family of growth factors may provide interesting opportunities for preventing and treating mycobacterial infections.

## Experimental procedures

### Organisms and media

*Micrococcus luteus* NCIMB 13267 ('Fleming strain 2665') was grown in LMM (Kaprelyants and Kell, 1993) at 30°C in conical flasks on an orbital shaker. *Mycobacterium bovis* (BCG) was obtained from the Central Institute for Scientific Research on Tuberculosis, Moscow, Russia. Cultures (3 ml) were grown at 37°C in 10 ml tubes with tightly fitting screw caps without shaking in Sauton's medium supplemented with ADC (Connell, 1994) and 0.05% Tween 80. *Mycobacterium smegmatis* mc<sup>2</sup>155 was grown aerobically at 37°C in Sauton's medium.

### Cell viability by plating

Dilutions in LMM (*M. luteus*) and Sauton's medium (*M. smegmatis*) were plated in triplicate on agar-solidified plates containing nutrient broth E (NBE – LabM) and incubated for 3 days at 30°C for *M. luteus*, or 37°C for *M. smegmatis*.

Dilutions of *M. bovis* (BCG) were made in Sauton's medium supplemented with 0.05% Tween 80, spread on agar-solidified Sauton's medium supplemented with ADC (Connell, 1994) and incubated at 37°C for 3–4 weeks. Suspensions of all organisms were passed 10 times through a 23-gauge needle to break up loose cell aggregates before dilution.

### Bacterial growth kinetics

For the experiment shown in Fig. 4A, *M. smegmatis* strains containing pAGH or pAGM0 were grown overnight to stationary phase in NBE containing 20 µg hygromycin ml<sup>-1</sup>. Under these conditions clumping is minimized. The cultures, which reached a final OD<sub>600</sub> of 2.1 (pAGH) and 2.4 (pAGM0), were diluted with Sauton's medium to an OD<sub>600</sub> of 1.0 and a further reduction in clumping was obtained by passing them ten times through a 23-gauge syringe needle. A sample was taken for cfu determination. Bacteria were then serially diluted and inoculated at three different densities into Sauton's medium (nutritional shift-down) supplemented with 0.05% Tween-80 in Bioscreen plates (five replicates for each strain at each density). Growth was at 37°C with constant shaking in a Bioscreen C optical growth analyser (Laboratory-systems, Finland). The data shown are the averages of readings from the five replicate cultures. The standard deviations on these measurements are smaller than the data points on the Figure. Similarly treated bacteria were used to inoculate the plates shown in Fig. 4B–E.

### Production of recombinant proteins

Recombinant Rpf was obtained as previously described (Mukamolova *et al.*, 1998). The five *rpf*-like genes from *M. tuberculosis* were amplified from H37Rv DNA using the following primer pairs (*Eco*RI, *Nco*I, *Nde*I and *Bam*HI restriction sites introduced for cloning purposes are in italics): Rv0867F (CCAGAATTCATATGGCTCAGGCGACCGCGGCCACC) + Rv0867R (TGGCGGATCCTATCAGCCGATGACGTACGCTG); Rv1009F (5'-GTGGCCATGGGCATATGGCAAGCAAAACGGTGACGTTGA3') + Rv1009R (5'-CAGCCGGATCCTCAGCGCACCCGCT-3'); Rv1884F (5'-TCCTGAATTCATATGGG TCCCAGCCCGAAGTGG-3') + Rv1884R (5'-CATGGGATC CGTCAGCGCGGAATACTTG-3'); Rv2389F (5'-ATCAGAA TCATATGGACGACATCGATTGGGACGC-3') + Rv2389R (5'-CGCAGGATCCCTCAATCGTCCCTGCTCC-3'); Rv2450F (5'-TGGAGAATTCATATGGACGACGCGGGCTTGA-3') + Rv2450R (5'-TCTTGGATCCTATCAGCCGCGGCGGCCGCA-3'). Amplification produced derivatives of each gene lacking their 5' ends, predicted to encode signal sequences or N-terminal *trans*-membrane helices/anchors (<http://www.cbs.dtu.dk/services/SignalP/> and <http://www.cbs.dtu.dk/services/TMHMM-1.0/>). In the case of RpfB (Rv1009), an additional C<sub>24</sub>S substitution was introduced. The truncated form of *rpf* was amplified from a previously cloned (Mukamolova *et al.*, 1998) 1375 bp segment of *M. luteus* DNA using primers TR1 (5'-GTCAGAATTCATATGGCCACCGTGGACACCTG-3') + TR2 (5'-TGACGGATCCTATTACAGCTTCTGCGAGCACAG-3'). Polymerase chain reaction products were first established in *E. coli* XL-2 blue as *Eco*RI-

*Bam*HI or *Nco*I-*Bam*HI fragments in pMTL20 (Chambers *et al.*, 1988) and their sequences verified. They were then cloned as *Nde*I-*Bam*HI fragments in pET19b (Novagen) and re-established in *E. coli* XL-2 blue. The polyhistidine-tagged proteins were expressed in *E. coli* HMS174 (DE3) and purified essentially as described (Mukamolova *et al.*, 1998) to single band purity by SDS-PAGE. The appearance of cell extracts is shown in Fig. 6C, from which it is clear that these proteins are subject to both aggregation and degradation when expressed as recombinant derivatives in *E. coli*. Anti-His tag antibodies (data not shown) also detect the various bands detected by the anti-Rpf antibodies (Fig. 6C). Except in the case of RpfB, additional purification was by Mono Q chromatography. RpfC (Rv1884c) and RpfD (Rv2389c) eluted as single peaks whereas RpfA (Rv0867c) and RpfE (Rv2450c) and *M. luteus* Rpf (full length and truncated form) were present in several fractions (revealed by immunoblotting) one of which only, was biologically active. The active fraction was used for experiments. Freshly isolated proteins (usually same day or next day) were used in all experiments, as activity is substantially reduced (1 log) during storage for 1 week at 20°C in 50% glycerol.

### Activity assay

Growth of *M. luteus* and *M. smegmatis* was monitored in a Bioscreen C growth analyser (Labsystems, Finland) using a 600 nm filter. Freshly prepared recombinant proteins diluted 1 : 100 in the appropriate growth medium were sterilised by filtration (0.22 µm, Gelman) and then serially diluted in growth medium either 30- or 10-fold, for assays with *M. luteus* and *M. smegmatis* respectively. After breaking up aggregates (see above), late log cultures (OD<sub>600</sub> = 3.5–4.0 for *M. luteus*; OD<sub>600</sub> = 3.0–3.5 for *M. smegmatis*) were serially diluted using growth medium. Samples (5 µl) of each dilution (five to ten replicates) were added to wells containing medium (200 µl), together with serially diluted protein. Incubation was at 30°C (*M. luteus*) or 37°C (*M. smegmatis*) with continuous shaking on the high setting. Measurements were taken hourly for 240 h. *Mycobacterium bovis* (BCG) bioassays were in tubes (see above) inoculated with cells (10<sup>2</sup>–10<sup>6</sup> cells per ml) from cultures of different ages (10 days–5 month). Incubation was at 37°C without shaking and growth was monitored by direct measurement (OD<sub>600</sub>) of the tube cultures or by plating.

### Antibody purification and Western blotting

Rabbits were immunized three times at 3-week intervals by subcutaneous injection with 1 ml of a 50% (v/v) mixture of Rpf (1 mg ml<sup>-1</sup> in water) and incomplete Freund's adjuvant (Sigma). Serum was collected 10 days after the last immunization and antibodies were purified by affinity chromatography [Rpf conjugated to CNBr-activated Sepharose 4B, (Sigma)]. Sheep antibodies to the truncated form of Rpf were obtained commercially (Micropharm, Newcastle Emlyn, UK) and purified as above. Both types of antibodies also detected recombinant versions of all five Rpf-like proteins of *M. tuberculosis*, as well as various aggregated forms and degradation products (see Fig. 6C for reactions to rabbit anti-Rpf antibody). The rabbit antibodies were used for the experiments

shown in Fig. 7, except the experiment in Fig. 7A, for which immunoglobulins purified from preimmune and immune serum using G-protein Sepharose (Pharmacia) were used.

For Western blotting, 1 ml of a 3-week-old culture of *M. bovis* (BCG) Russian strain, grown in Sauton's medium supplemented with ADC and 0.05% Tween-80, was washed twice with Sauton's medium to remove traces of ADC and inoculated into 100 ml Sauton's medium in a 500 ml flask. Growth was for 3 weeks at 37°C without shaking. For *M. smegmatis*, growth was for 16 h (overnight) with shaking in Sauton's medium (lacking ADC and Tween-80). A protease inhibitor cocktail was added to the cultures 30 min before harvesting and incorporated into all buffer solutions, according to the manufacturer's instructions (Roche). After centrifugation, culture supernatants were filtered (0.22 µm) and proteins present were concentrated using DEAE-Sepharose 6B and CM cellulose column chromatography. The DEAE-Sepharose 6B fast flow column, equilibrated with buffer A (20 mM TrisHCl, pH 7.5; 20 mM KCl, 1 mM EDTA, 1 mM DTT) bound all detectable Rpf-like proteins. The column was washed with 5 × vol buffer A and eluted with 3 × vol buffer B (buffer A containing 1 M NaCl). Samples (1 ml) of the eluate were precipitated with 10% TCA, washed twice with acetone, dried, reconstituted in 20 µl loading buffer and used for SDS gel electrophoresis/blotting. Pre-stained size standards were from BioRad (cat no. 161–0305). For the experiments shown in Fig. 6, rabbit anti-Rpf antibodies were used; similar data were obtained with the sheep anti-truncated Rpf antibodies.

### Confocal microscopy

Cells from early logarithmic phase ( $OD_{600} = 0.25$ ) grown in Sauton's medium supplemented with ADC and 0.05% Tween-80, were centrifuged, washed with PBS and fixed in 0.1% glutaraldehyde in PBS for 30 min at 37°C. After washing with PBS, cells were incubated in PBS containing 5% BSA for 30 min at 37°C with shaking, before treatment with sheep anti-truncated Rpf antibodies at 1 : 1000 dilution in PBS containing 2% BSA. After incubation for 1 h at 37°C with shaking, cells were washed three times with PBS, containing 0.2% Tween-80. The bacteria were then incubated in PBS, containing 2% BSA and FITC-conjugated donkey anti-sheep IgG antibodies at a 1 : 500 dilution (Sigma, cat. no. F7634). After washing (as described above) cells were examined using a BioRad MRC1024ES confocal microscope with excitation at 488 nm (100 mW argon laser).

### Effect of anti-Rpf antibodies on bacterial growth

*Mycobacterium bovis* (BCG) ( $10^2$  cfu from a 2-week-old culture or  $10^6$  cfu from a 6-week-old culture) and *M. tuberculosis* Academia strain ( $10^5$  cfu from a 2-month-old culture) were inoculated into 3 ml Sauton's medium supplemented with ADC and 0.05% Tween-80. Rabbit anti-Rpf antibodies were added and growth was monitored by measuring the  $OD_{600}$ . For the experiment shown in Fig. 7C, a 20 ml culture was established in a 100 ml conical flask without shaking and growth was monitored by plating on agar-solidified Sauton's medium supplemented with ADC.

### Expression of rpf in *M. smegmatis*

The *rpf* gene was introduced into *M. smegmatis* under the control of the  $P_{ami}$  promoter (Parish *et al.*, 1997) in plasmid pAGM0. The construction of this plasmid and the vector, pAGH, from which it is derived, was described by (Mukamolova *et al.* (2002). Plasmid pAGM1 is similar to pAGM0 except that  $P_{ami}$  generates a counter-transcript of *rpf*.

### Reverse transcriptase-PCR

Specific primer pairs were designed for each of the five *rpf*-like genes of *M. tuberculosis* (the corresponding regions of the cognate genes in *M. bovis* are identical): RT0867F (5'-TATGAGTGGACGCCACCGTAA-3') + RT0867R (5'-ACTGC AAGCCACCGAGGTAAC3'); RT1009F (5'-AGGACCCGGAG ATGAACATGA3') + RT1009R (5'-GCACACCACCGTAATAC CCGT-3'); RT1884F (5'-GCTTCTCGGGAACAACAAATC-3') + RT1884R (5'-CGGAATACTTGCCTGAATGCC-3'); RT 2389F (5'-GCTATGACACCGGGTTTGCTT-3') + RT2389R (5'-GCAGACCACCGTATAACCCGT-3'); RT2450F (5'-GT TGAAGAACGCCCCGTACGAC-3') + RT2450R (5'-TTACCG GTGTTGATCGACCAG-3'). RNA was prepared from 1 ml culture samples of *M. bovis* (BCG) ( $OD_{600} = 0.3$ ) using the RNeasy Mini Kit (Qiagen) and treated twice or three times with 10 U of RNAase-free DNAase I (Roche) for 30 min. The RNA from exponentially growing cells of *M. tuberculosis* H37Rv was generously provided by P. Butcher and J. Mangan. Reverse transcription (25 µl) reactions contained 2 µg RNA, 1 µg of the relevant reverse primer, 40 U RNasin ribonuclease inhibitor (Promega) and 30 U AMV reverse transcriptase (Promega). Reactions (1 h) were performed at 60°C in the presence of 10% DMSO for templates containing 71–74% G + C (RpfA, RpfC and RpfD) and 1 M betaine for templates containing 83–85% G + C (RpfB and RpfE). Reactions were terminated by incubation at 75°C for 5 min. Control reactions, lacking AMV reverse transcriptase were performed simultaneously. For PCR reactions, 2 µl samples of the RT reaction products were used as template in the presence of both primers. Samples were denatured for 5 min at 94°C followed by a single cycle of 30 s at 94°C, 30 s at 52°C, 60 s at 72°C and then 29 cycles of 30 s at 94°C, 60 s at 72°C. No PCR product was produced in any of the control reactions in which reverse transcriptase had been omitted from the previous step, nor when a treatment with RNAase preceded the initial reverse transcription step.

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## References

- Aldovini, A., Husson, R.N., and Young, R.A. (1993) The *uraA* locus and homologous recombination in *Mycobacterium bovis* BCG. *J Bacteriol* **175**: 7282–7289.
- Arruda, S., Bomfim, G., Knights, R., Huimabyron, T., and Riley, L.W. (1993) Cloning of a *Mycobacterium tuberculosis* DNA fragment associated with entry and survival inside cells. *Science* **261**: 1454–1457.
- Bateman, A., and Bycroft, M. (2000) The structure of a LysM domain from *E. coli* membrane-bound lytic murein transglycosylase D (MltD). *J Mol Biol* **299**: 1113–1119.
- Behr, M.A., Wilson, M.A., Gill, W.P., Salamon, H., Schoolnik, G.K., Rane, S., and Small, P.M. (1999) Comparative genomics of BCG vaccines by whole-genome DNA microarray. *Science* **284**: 1520–1523.
- Biketov, S., Mukamolova, G.V., Potapov, V., Gilenkov, E., Vostroknutova, G., Kell, D.B., Young, M., and Kaprelyants, A.S. (2000) Culturability of *Mycobacterium tuberculosis* cells isolated from murine macrophages: a bacterial growth factor promotes recovery. *FEMS Immunol Med Microbiol* **29**: 233–240.
- Bloom, B.R., and Murray, C.J.L. (1992) Tuberculosis – commentary on a reemergent killer. *Science* **257**: 1055–1064.
- Callard, R., and Gearing, A. (1994) *The Cytokine Facts Book*. London: Academic Press.
- Chambers, S.P., Prior, S.E., Barstow, D.A., and Minton, N.P. (1988) The pMTL nic- cloning vectors. 1. Improved pUC polylinker regions to facilitate the use of sonicated DNA for nucleotide sequencing. *Gene* **68**: 139–149.
- Clewell, D.B. (1993) Bacterial sex pheromone-induced plasmid transfer. *Cell* **73**: 9–12.
- Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., et al. (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**: 537–544.
- Connell, N.D. (1994) *Mycobacterium*: isolation, maintenance, transformation, and mutant selection. *Methods Cell Biol* **45**: 107–125.
- Dubos, R.J., and Davis, B.D. (1946) Factors affecting the growth of tubercle bacilli in liquid media. *J Exp Med* **83**: 409–423.
- Dye, C., Scheele, S., Dolin, P., Pathania, V., and Raviglione, R.C. (1999) Consensus statement. Global burden of tuberculosis – estimated incidence, prevalence, and mortality by country. *WHO Global Surveillance Monitoring Project JAMA* **282**: 677–686.
- Flynn, J.L., and Chan, J. (2001) Tuberculosis: latency and reactivation. *Infect Immun* **69**: 4195–4201.
- Franklin, T.J. (1980) Binding energy and the activation of hormone receptors. *Biochem Pharmacol* **29**: 853–856.
- Fuqua, C., and Greenberg, E.P. (1998) Self perception in bacteria: quorum sensing with acylated homoserine lactones. *Curr Opin Microbiol* **1**: 183–189.
- Fuqua, W.C., Winans, S.C., and Greenberg, E.P. (1994) Quorum sensing in bacteria the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J Bacteriol* **176**: 269–275.
- Fuqua, C., Winans, S.C., and Greenberg, E.P. (1996) Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. *Annu Rev Microbiol* **50**: 727–751.
- Gero, A. (1983) Desensitization, two-state receptors and pharmacological parameters. *J Theoret Biol* **103**: 137–161.
- Gomez, L., Johnson, S., and Gennaro, M.L. (2000) Identification of secreted proteins of *Mycobacterium tuberculosis* by a bioinformatic approach. *Infect Immun* **68**: 2323–2327.
- Hernandez-Pando, R., Jeyanathan, M., Mengistu, G., Aguilar, D., Orozco, H., Harboe, M., Rook, G.A.W., and Bjune, G. (2000) Persistence of DNA from *Mycobacterium tuberculosis* in superficially normal lung tissue during latent infection. *Lancet* **356**: 2133–2138.
- Höner zu Bentrup, K., and Russell, D.G. (2001) Mycobacterial persistence: adaptation to a changing environment. *Trends Microbiol* **9**: 597–605.
- Horinouchi, S., and Beppu, T. (1994) A-factor as a microbial hormone that controls cellular differentiation and secondary metabolism in *Streptomyces griseus*. *Mol Microbiol* **12**: 859–864.
- Kaiser, D., and Losick, R. (1993) How and why bacteria talk to each other. *Cell* **73**: 873–885.
- Kaprelyants, A.S., and Kell, D.B. (1993) Dormancy in stationary-phase cultures of *Micrococcus luteus*: flow cytometric analysis of starvation and resuscitation. *Appl Environ Microbiol* **59**: 3187–3196.
- Kaprelyants, A.S., and Kell, D.B. (1996) Do bacteria need to communicate with each other for growth? *Trends Microbiol* **4**: 237–242.
- Kaprelyants, A.S., Gottschal, J.C., and Kell, D.B. (1993) Dormancy in non-sporulating bacteria. *FEMS Microbiol Rev* **104**: 271–286.
- Kaprelyants, A.S., Mukamolova, G.V., and Kell, D.B. (1994) Estimation of dormant *Micrococcus luteus* cells by penicillin lysis and by resuscitation in cell-free spent medium at high dilution. *FEMS Microbiol Lett* **115**: 347–352.
- Kaprelyants, A.S., Mukamolova, G.V., Kormer, S.S., Weichart, D.H., Young, M., and Kell, D.B. (1999) Intercellular signalling and the multiplication of prokaryotes: bacterial cytokines. *Symp Soc Gen Microbiol* **57**: 33–69.
- Kell, D.B., Kaprelyants, A.S., and Grafen, A. (1995) Pheromones, social behaviour and the functions of secondary metabolism in bacteria. *Trends Ecol Evol* **10**: 126–129.
- Kell, D.B., and Young, M. (2000) Bacterial dormancy and culturability: the role of autocrine growth factors. *Curr Opin Microbiol* **3**: 238–243.
- Kell, D.B., Kaprelyants, A.S., Weichart, D.H., Harwood, C.L., and Barer, M.R. (1998) Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. *Antonie Van Leeuwenhoek* **73**: 169–187.
- Kleerebezem, M., Quadri, L.E.N., Kuipers, O.P., and de Vos, W.M. (1997) Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria. *Mol Microbiol* **24**: 895–904.
- Lazazzera, B.A., and Grossman, A.D. (1998) The ins and outs of peptide signaling. *Trends Microbiol* **6**: 288–294.
- Lim, A., Eleuterio, M., Hutter, B., Murugasu-Oei, B., and Dick,

- T. (1999) Oxygen depletion-induced dormancy in *Mycobacterium bovis* BCG. *J Bacteriol* **181**: 2252–2256.
- McCune, R.M., Feldman, F.M., Lambert, H., and McDermott, W. (1966) Microbial persistence. I. The capacity of tubercle bacilli to survive sterilization in mouse tissues. *J Exp Med* **123**: 224–268.
- McDermott, W. (1958) Microbial persistence. *Yale J Biol Med* **30**: 257.
- Manganelli, R., Voskuil, M.I., Schoolnik, G.K., and Smith, I. (2001) The *Mycobacterium tuberculosis* ECF sigma factor  $\sigma^E$ : role in global gene expression and survival in macrophages. *Mol Microbiol* **41**: 423–437.
- Mukamolova, G.V., Kormer, S.S., Yanopolskaya, N.D., and Kaprelyants, A.S. (1995) Properties of dormant cells in stationary-phase cultures of *Micrococcus luteus* during prolonged incubation. *Microbiology* **64**: 284–288.
- Mukamolova, G.V., Kaprelyants, A.S., Young, D.I., Young, M., and Kell, D.B. (1998) A bacterial cytokine. *Proc Natl Acad Sci USA* **95**: 8916–8921.
- Mukamolova, G.V., Kormer, S.S., Kell, D.B., and Kaprelyants, A.S. (1999) Stimulation of the multiplication of *Micrococcus luteus* by an autocrine growth factor. *Arch Microbiol* **172**: 9–14.
- Mukamolova, G.V., Turapov, O.A., Kazaryan, K., Telkov, M., Kaprelyants, A.S., Kell, D.B., and Young, M. (2002) The *rpf* gene of *Micrococcus luteus* encodes an essential secreted growth factor. *Mol Microbiol* **46**: 611–621.
- Ohnishi, Y., Kameyama, S., Onaka, H., and Horinouchi, S. (1999) The A-factor regulatory cascade leading to streptomycin biosynthesis in *Streptomyces griseus*: identification of a target gene of the A-factor receptor. *Mol Microbiol* **34**: 102–111.
- Pai, S.R., Actor, J.K., Sepulveda, E., Hunter, R.L. Jr and Jagannath, C. (2000) Identification of viable and non-viable *Mycobacterium tuberculosis* in mouse organs by directed RT-PCR for antigen 85B mRNA. *Microb Pathog* **28**: 335–342.
- Parish, T., Mahenthalingam, E., Draper, P., Davis, E.O., and Colston, M.J. (1997) Regulation of the inducible acetamidase gene of *Mycobacterium smegmatis*. *Microbiology* **143**: 2267–2276.
- Parrish, N.M., Dick, J.D., and Bishai, W.R. (1998) Mechanisms of latency in *Mycobacterium tuberculosis*. *Trends Microbiol* **6**: 107–112.
- Salmond, G.P.C., Bycroft, B.D., Stewart, G.S.A.B., and Williams, P. (1995) The bacterial 'enigma': cracking the code of cell-cell communication. *Mol Microbiol* **16**: 615–624.
- Sherman, D.R., Voskuil, M., Schnappinger, D., Liao, R., Harrell, M.I., and Schoolnik, G.K. (2001) Regulation of the *M. tuberculosis* hypoxic response gene alpha-crystallin. *Proc Natl Acad Sci USA* **98**: 7534–7539.
- Shleeva, M.O., Bagramyan, K., Telkov, M.V., Mukamolova, G.V., Young, M., Kell, D.B., and Kaprelyants, A.S. (2002) Formation and resuscitation of 'non-culturable' cells of *Rhodococcus rhodochrous* and *Mycobacterium tuberculosis* in prolonged stationary phase. *Microbiology* **148**: 1581–1591.
- Sun, Z., and Zhang, Y. (1999) Spent culture supernatant of *Mycobacterium tuberculosis* H37Ra improves viability of aged cultures of this strain and allows small inocula to initiate growth. *J Bacteriol* **181**: 7626–7628.
- Votyakova, T.V., Kaprelyants, A.S., and Kell, D.B. (1994) Influence of viable cells on the resuscitation of dormant cells in *Micrococcus luteus* cultures held in extended stationary phase. The population effect. *Appl Env Microbiol* **60**: 3284–3291.
- Wayne, L.G. (1960) The bacteriology of resected tuberculous pulmonary lesions. II. Observations on bacilli which are stainable but which cannot be cultured. *Am Rev Resp Dis* **82**: 370–377.
- Wayne, L.G. (1984) Mycobacterial speciation. In *The Mycobacteria: a Sourcebook*. Kubica, G.P., and Wayne, L.G. (eds). New York: Marcel Dekker, pp. 25–65.
- Wayne, L.G. (1994) Dormancy of *Mycobacterium tuberculosis* and latency of disease. *Eur J Clin Microbiol Infect Dis* **13**: 908–914.
- Wayne, L.G., and Sohaskey, C.D. (2001) Nonreplicating persistence of *Mycobacterium tuberculosis*. *Annu Rev Microbiol* **55**: 139–163.
- de Wit, D., Wootton, M., Dhillon, J., and Mitchison, D.A. (1995) The bacterial DNA content of mouse organs in the Cornell model of dormant tuberculosis. *Tuber Lung Dis* **76**: 555–562.
- Young, D.B., and Duncan, K. (1995) Prospects for new interventions in the treatment and prevention of mycobacterial disease. *Annu Rev Microbiol* **49**: 641–673.
- Yuan, Y., Crane, D.D., and Barry, C.E. III (1996) Stationary phase-associated protein expression in *Mycobacterium tuberculosis*: function of the mycobacterial alpha-crystallin homolog. *J Bacteriol* **178**: 4484–4492.
- Zhang, Y., Yang, Y., Woods, A., Cotter, R.J., and Sun, Z. (2001) Resuscitation of dormant *Mycobacterium tuberculosis* by phospholipids or specific peptides. *Biochem Biophys Res Commun* **284**: 542–547.





# Resuscitation factors from mycobacteria: homologs of *Micrococcus luteus* proteins<sup>☆</sup>

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## KEYWORDS

Resuscitation factor;  
Growth promotion;  
Dormancy

**Summary Setting:** Resuscitation promoting factors (Rpfs) are proteins, originally identified in *Micrococcus luteus*, that promote recovery of bacteria from a viable but non-replicating phase (e.g., stationary phase or latency) to a replicating phase. Purified *M. luteus* Rpf can stimulate growth and increase recovery of *M. luteus* bacteria as well as *Mycobacterium tuberculosis* bacteria from prolonged stationary cultures.

**Objective:** To clone and characterize Rpfs from mycobacteria.

**Design:** We cloned one *M. avium* subsp. *paratuberculosis* *rpf* gene and one *M. tuberculosis* *rpf* gene into the pET19b or pET21a vector for expression in *Escherichia coli*. The His-tag recombinant proteins were purified and characterized.

**Results:** When the purified recombinant proteins were added to Sauton medium (a relatively minimal medium) at 100–500 pM, lag phase for mycobacteria from non-replicating cultures was shortened and there was a 10- to 100-fold increase in colony-forming units compared with control samples. In most probable number assays, the mycobacterial Rpfs increased recovery of mycobacteria from late stationary culture by about 10-fold. The Rpfs also promoted recovery of extensively washed *Mycobacterium smegmatis* bacteria inoculated into Sauton medium. Rpfs had only minor effects on growth of *M. tuberculosis* in BACTEC 12B broth, a rich medium.

**Conclusion:** The mycobacterial Rpfs demonstrate resuscitation activities similar to those of the *M. luteus* Rpf.

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## Introduction

Mycobacteria are important human and animal pathogens and cause a broad range of diseases from superficial skin infections to the devastating diseases of tuberculosis and leprosy. Among infectious diseases, tuberculosis (TB) remains the second leading killer of adults in the world, with more than 2 million TB-related deaths each year

and more than 8 million new cases.<sup>1,2</sup> *Mycobacterium avium* subsp. *paratuberculosis* is the causative agent of Johne's disease in ruminants.<sup>3</sup> This mycobacterium has also been sporadically isolated from patients affected with Crohn's disease (CD).<sup>4,5</sup> CD is a chronic inflammatory gastrointestinal disease which often affects young people and persists through life. It is estimated that CD afflicts more than 500,000 persons in the United States. The prevalence of CD appears to be increasing in the US and other parts of the world. Although it has been well established that *M. avium* subsp. *paratuberculosis* is a cause of Johne's disease in cattle, goats, sheep and other ruminants, a role for this mycobacterium in the pathogenesis of CD remains to be determined.

<sup>☆</sup>Use of trade names is for identification only and does not constitute endorsement by the US Department of Health and Human Services, the Public Health Services, or the Centers for Disease Control and Prevention.

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Efforts to combat mycobacterial diseases are hindered by an inadequate understanding of their underlying pathogenesis and physiology. Culture of mycobacterial pathogens from patients is the standard method for diagnosing mycobacteria-caused diseases. However, the slow growth of these organisms often results in a 3–6 week delay before culture data for *M. tuberculosis* are available and 3–6 months for *M. avium* subsp. *paratuberculosis*. In addition, the recovery of colony-forming units (CFUs) has often been less than anticipated from microscopic observations of acid-fast bacilli. This raises the possibility that dormant or non-replicating cells may be present. And such cells may be viable but not able to form colonies on plates or grow in liquid media.

Studies on the recovery of *Micrococcus luteus* bacteria from a non-replicating phase led to the discovery of a resuscitation promoting factor (Rpf) which can promote the recovery of dormant bacteria. Early studies demonstrated that supernatant from spent medium of *M. luteus* cultures increased the number of viable cells recovered from dormant *M. luteus* cultures.<sup>6–8</sup> The resuscitation factor is secreted into culture medium when the *M. luteus* bacteria enter stationary phase.<sup>9,10</sup> The gene encoding the *M. luteus* Rpf has been cloned and expressed in *E. coli*.<sup>10</sup> The addition of purified Rpf to liquid medium at picomolar concentrations greatly increased the number of viable cells that could be recovered from stationary-phase cultures and shortened the lag-phase.<sup>10,11</sup> Purified Rpf from *M. luteus* can also shorten lag-phase and increase the recovery of *M. tuberculosis*, *M. avium* and other mycobacteria from prolonged stationary cultures.<sup>10–13</sup> Interestingly, the *M. luteus* RPF shares homology with several mycobacterial open reading frames (ORFs).<sup>10,14</sup>

Previous studies have raised the possibility of Rpfs in mycobacteria. Sun and Zhang<sup>15</sup> showed that the addition of supernatant from early stationary cultures of *M. tuberculosis* H37Ra could increase the viability of aged cultures. The resuscitation activity disappeared when the supernatant was treated with heat and acid. An 8-kDa protein was subsequently recovered from culture supernatants that had resuscitation activity.<sup>16</sup> Synthetic peptides derived from this protein could resuscitate dormant bacilli from a 1-year-old culture that were unable to form colonies when plated directly onto solid media. Recently, five genes encoding Rpf homologs from *M. tuberculosis* were cloned and expressed in *E. coli*, and the products of each of the five *rpf* genes were shown to have resuscitation activity and to stimulate bacterial growth.<sup>14</sup>

In the study reported here, we cloned and expressed one *Rpf*-like gene (*mptb*) from *M. avium* subsp. *paratuberculosis* and one (*Rv1009*) from *M. tuberculosis*. These gene products stimulated the growth of mycobacteria and enhanced the recovery of replicating cells from non-replicating phases. Overall, the data indicate that the product of the *M. avium* subsp. *paratuberculosis* *rpf*-like gene has resuscitation activity and confirm the recent report that *M. tuberculosis* Rv1009 has resuscitation activity.<sup>14</sup>

## Material and methods

### Strains and media

*M. tuberculosis* H37Rv, *M. avium* subsp. *paratuberculosis* ATCC43015, and *M. smegmatis* 607 bacteria were routinely maintained in Middlebrook 7H9 medium containing 0.05% Tween80 and ADC (albumin-dextrose-catalase) enrichment (Becton Dickinson and Company, Sparks, MD) unless otherwise noted. *M. avium* subsp. *paratuberculosis* cultures were supplemented with 2 mg of ferric mycobactin J (Allied Monitor Inc., Fayette, MO) per liter. Mycobacteria from cultures varying in age from 1 to 4 months were used for growth and resuscitation experiments. *Escherichia coli* bacteria were routinely maintained in Luria-Bertani (LB) medium.<sup>17</sup> *E. coli* strain DH5 $\alpha$  (Life Technologies Inc., Rockville, MD) was used for DNA manipulation and *E. coli* strain BL21(DE3) (Novagen, Madison, CA) was used for protein expression. Sauton medium was prepared as described elsewhere.<sup>18</sup>

### Construction of plasmids

Plasmid DNA preparation, restriction endonuclease analysis and ligations were carried out by standard methods described in Sambrook et al.<sup>17</sup> Polymerase chain reaction (PCR) amplifications were performed by using Pfu DNA polymerase (Stratagene, La Jolla, CA) according to manufacturer's instructions. Oligonucleotides were synthesized by the CDC core facility. A 660-bp product (contig 1289, as of May 5, 2002; TIGR Microbial Database) was amplified from *M. avium* subsp. *paratuberculosis* genomic DNA using primers RPF/PTF7 5'-CGCA-TATGGGCCAGGCGGCCGCGGCCACCGACGGC and RPF/PTBamH1 5'-ACGGATCCTATTAGGCGCTGGGTG-CGGGCTGCAC, which has a *Bam*HI site at the end. Each PCR reaction was prepared in a total volume of 50  $\mu$ l including 2  $\mu$ l dimethyl sulfoxide (DMSO), 8  $\mu$ l of 1.25 mM dNTP (Amersham Biosciences, Piscataway, NJ), 1  $\mu$ l of each primer at 20  $\mu$ M, 2  $\mu$ l

of DNA (equaling 25–250 ng), 5  $\mu$ l of 10  $\times$  Pfu buffer, 1  $\mu$ l of Pfu enzyme, and 30  $\mu$ l of H<sub>2</sub>O. PCR reactions were performed in a Gene AMP PCR System 9700 (PE Applied Biosystems, Foster City, CA) with reaction cycle: an initial denaturation step of 2 min at 94°C, followed by 30 cycles of 1 min at 94°C, 30 s at 60°C, and 1 min 72°C. After amplification, poly(A) overhangs were generated by adding 1  $\mu$ M of dATP (Amersham Biosciences) and 1  $\mu$ l of Taq polymerase (PE Applied Biosystems) and by incubating at 72°C for 15 min. The 660-bp amplicon was cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) to generate pMIN26. The nucleotide sequence of the cloned insert was determined using a CEQ 2000 DNA sequencer and the dye terminator cycle sequencing kit (Beckman Coulter, Fullerton, CA). The gene was subcloned into the pET19b expression vector (Novagen) utilizing *Nde*I/*Bam*HI restriction sites to generate pMIN27. pMIN27 was transformed into *E. coli* BL21(DE3) (Novagen) for protein expression studies.

One *M. tuberculosis* *rpf* gene (*Rv1009*, Acc#e2052146) was amplified from genomic DNA using primers RPFTB3, 5'-CGCATATGGCATG-CAAAACGGTGACGTTGACCGTC-3', and RPFTBR4, 5'-CGCTCGAGGCGCGCACCCGCTCGTGACGACATAC-3', as described above. The amplicon was cloned into pCR2.1-TOPO (Invitrogen) to generate pMIN2. The 1 kb *Nde*I/*Xho*I fragment of pMIN2 was cloned into pET21a to generate pMIN9.

### Expression and purification of Rpf

The *E. coli* BL21(DE3) strains carrying pMIN9 and pMIN27 were grown in LB medium containing 100 mg of ampicillin (Sigma, St. Louis, MO) per liter overnight at 37°C. The overnight cultures were diluted 1:100 into 1 l of fresh LB-ampicillin medium. When the culture reached mid-log phase ( $OD_{600} = 0.5$ ), expression of the cloned inserts was induced by addition of isopropyl-1-thiol-(d)-galactopyranoside (IPTG) (Invitrogen) to a final concentration of 1 mM. After incubation at 37°C for 4–5 h, the bacteria were harvested by centrifugation at 5000g for 10 min at 4°C. Cells were lysed by sonication in 50 mM Tris-HCl (pH 7.5) containing 1 mM phenylmethanesulfonyl fluoride (Sigma). The sonicate was centrifuged at 7000g for 10 min and the soluble fraction was retained for protein purification.

Purification of the His-tagged proteins was performed according to the manufacturer's instructions (Novogen). Briefly, the soluble fraction was applied to a His-tag column (Novogen) previously equilibrated with 1x binding buffer. The column was washed with 8 volumes of 1x binding buffer and 5

volumes of 1x washing buffer. The protein was eluted with 1x elution buffer. The peak fractions were pooled and dialyzed against 10 mM Tris-HCl buffer (pH 7.5) containing 20% glycerol. The purity of protein was determined on an SDS-PAGE gel.<sup>17</sup> The Rpf solution was sterilized using a 0.22- $\mu$ m Millex®-GV filter (Millipore, Bedford, MA), aliquoted and stored at –80°C. Protein concentration was determined using the BIO-RAD DC protein assay (BIO-RAD, Hercules, CA).

### Growth measurement

To prepare bacterial suspensions, bacteria were harvested by centrifugation, washed 1 or 2 times in Sauton medium, and resuspended in Sauton medium. To disperse clumps of mycobacteria, 3-mm glass beads (Kimble Glass Inc, Vineland, NJ) were added (about one-fifth volume) and the cell suspensions with glass beads vortexed for 1 min. The cells were diluted and appropriate dilutions inoculated into medium. Mycobacterial growth was assayed either by turbidity ( $OD_{600}$ ) measurements or by counting CFUs on Sauton agar plates.

### Bacterial counts

The bacterial suspension for total cell counts was prepared as described above. The bacteria were counted microscopically with a Petroff Hausser Counting Chamber (Horsham, PA).

### MPN assay

Most probable number (MPN) assays were performed using a 3-month-old culture of *M. tuberculosis* bacteria. The mycobacteria were recovered by centrifugation, the supernatant was discarded, and the cells were resuspended in Sauton media. The suspensions were treated by vortexing with 3-mm glass beads to disperse clumps as described above. The bacterial suspensions were serially diluted such that only a few viable cells were present and portions added to 10 replicate tubes containing Sauton medium. Rpf was added to 5 of the 10 tubes; the 5 remaining tubes without Rpf served as the control. After 40 days of incubation at 37°C, the number of tubes with visible growth was recorded and MPN values were calculated based on the Meynell's table.<sup>19</sup>

### BACTEC culture assay

Suspensions of *M. tuberculosis* bacteria were prepared from 1- to 3-month-old cultures as

described above. Bacteria were diluted in Middlebrook 7H9 medium, and 0.4 ml of the dilution (approximately  $10^2$ – $10^4$  cells/ml) was inoculated into BACTEC<sup>®</sup> 12B mycobacterial culture vials (Becton Dickinson and Company). Rpf-tb (final concentrations, 16, 64, 256 pM and 1 nM and 5 nM) was added to the cultures, which were then incubated at 37°C without aeration. The growth of the culture was measured daily with the BACTEC-TB 460 system (Becton Dickinson and Company).

## Results

### Identification of mycobacterial ORFs that are homologous to *M. luteus* Rpf

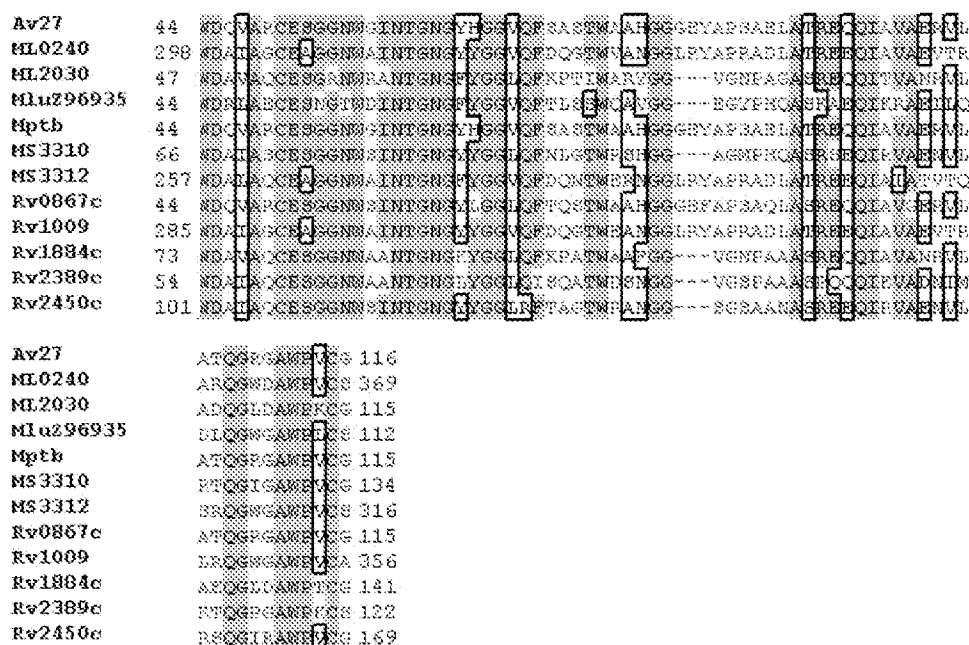
The available genome sequences of several *Mycobacterium* species were searched to identify gene products homologous to the Rpf of *M. luteus*. The search identified 19 mycobacterial ORFs which shared significant homology with the *M. luteus* RPF: 5 ORFs of *M. tuberculosis* (Rv0867c, Rv1009, Rv1884c, Rv2389c, Rv2450c), 3 ORFs of *M. leprae* (ML0250, ML2030, ML2151), 5 ORFs of *M. bovis*, 2 ORFs of *M. smegmatis* (genome unfinished), 2 ORFs of *M. avium* and 2 ORFs of *M. avium* subsp. *paratuberculosis* (genome unfinished). Figure 1 shows partial amino acid alignments of putative mycobacterial Rpf and the *M. luteus* Rpf. In

addition to sharing the conserved domains with the *M. luteus* Rpf, the mycobacterial ORFs share significant homology with each other. For example, Rv0867c shares 63% identity in a 200 amino acid overlap with ML2151 and 70% identity in a 257 amino acid overlap with Av27 (contig 27) and Mptb (contig 1289, as of May 5, 2002; TIGR Microbial Database). Rv1009 shares 43% homology with Rv2450c in a 147 amino acid overlap and Rv1884c shares 55.4% identity with Rv2389c in a 101 amino acid overlap.

In the genome annotations, the functions of these mycobacterial gene products are not assigned and they are classified as conserved hypothetical proteins. Rpf from *M. luteus* is a secreted protein.<sup>12</sup> Analysis of the mycobacterial Rpf-like products using the SMART program (<http://smart.embl-heidelberg.de/>) indicates that each has a hydrophobic amino acid cluster indicative of a signal sequence or transmembrane helix. This suggests these proteins might be found in an extracytoplasmic (Rv1009) or extracellular location (Rv0867c, Rv1884c, Rv2389c, Rv2450c, Av27, and Mptb). Rv1009 also contains a prokaryotic membrane lipoprotein lipid attachment site.

### Expression and purification of recombinant mycobacterial Rpf

To investigate the function of the *M. avium* subsp. *paratuberculosis* Rpf homologue, a 0.66-kb DNA

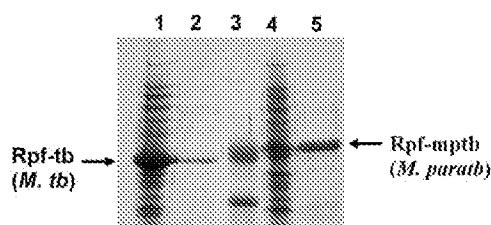


**Figure 1** Partial amino acid sequence alignments of putative Rpf. Mlu296935 is the *M. luteus* Rpf. Rv0867c, Rv1009, Rv1884c, Rv2450c are predicted proteins from *M. tuberculosis*. ML0240 and ML2030 are from *M. leprae*. Av27 is from *M. avium* contig27. Mptb is from *M. avium* subsp. *paratuberculosis* contig1289. MS3310 and MS3312 are from *M. smegmatis*. Black letters with shadow indicate identical residues; boxed letters indicate similar residues.

fragment excluding the predicted secretion signal sequence of the protein was PCR-amplified from genomic DNA using primers RPF/PTF7 and RPF/PTBAMHI. The identity of the PCR product was confirmed by DNA sequencing and it was subcloned into pET19b for protein expression. Using the same strategy, a 1-kb DNA fragment of one of the *M. tuberculosis* *rpf* genes (*Rv1009*) without its secretion signal sequence was PCR amplified from *M. tuberculosis* genomic DNA and cloned into pET21a for expression. Overexpression of the two cloned genes in *E. coli* produced soluble proteins which appeared as distinct bands on SDS-PAGE (Fig. 2). The expressed proteins were purified by His-tag column chromatography. The size of the recombinant *Rv1009* gene product was 40 kDa as expected. But the size of the recombinant *M. avium* subsp. *paratuberculosis* gene product observed on the polyacrylamide gel was 43 kDa, significantly larger than the predicated size (28 kDa). However, nucleotide sequencing of the cloned DNA and protein sequencing of the amino terminus of the recombinant protein indicated the expressed protein had the correct amino acid sequences. The unexpected apparent size may be related to the high proline (16.8%), alanine (19.5%), and glycine (12%) content of the predicted protein. Following convention, the products of *M. avium* subsp. *paratuberculosis* gene and *M. tuberculosis* gene *Rv1009* were designated as Rpf-mptb and Rpf-tb, respectively. Attempts to express *M. tuberculosis* genes *Rv0867c*, *Rv2389c* and *M. avium* *Av27* using a similar strategy were unsuccessful. Hence, our analysis focused on Rpf-mptb and Rpf-tb.

### Effect of Rpfs on the growth of mycobacteria

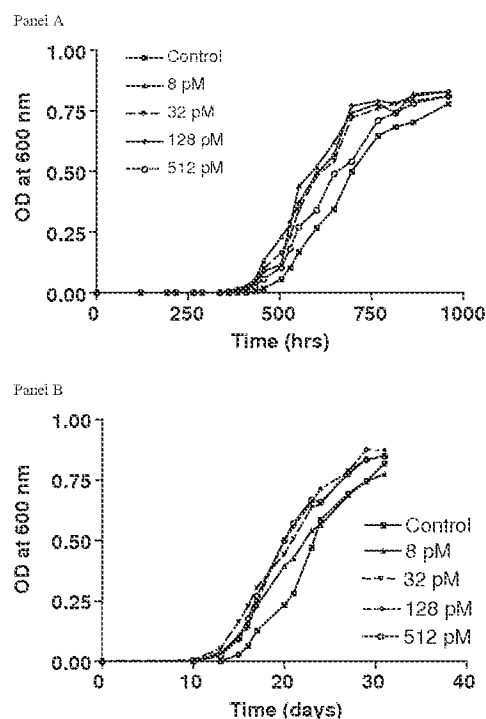
To generate mycobacteria that were in a viable but non-replicating phase, cultures that had been



**Figure 2** SDS-PAGE analysis of recombinant mycobacterial Rpfs. Lane 1, whole-cell lysate from *E. coli* expressing the *Rv1009* gene; lane 2, purified recombinant product (Rpf-tb); lane 3, molecular size markers, 43 kDa (top band), 38 kDa (lower band); lane 4, whole-cell lysate of *E. coli* expressing the *Mptb* gene; lane 5, purified recombinant Rpf-mptb.

maintained for greater than 1 month were used. The quiescent mycobacteria were harvested from such cultures, washed with Sauton media, and resuspended ( $\sim 10^2$  bacteria/ml) in Sauton medium with or without Rpf. The final concentration of the recombinant Rpfs varied from 8 to 512 pM. Bacterial growth was followed by optical density.

The Rpf-mptb significantly shortened the lag phase and enhanced the growth of the quiescent *M. avium* subsp. *paratuberculosis* bacteria in a dose-dependent manner (Fig. 3a). The optimal concentrations of Rpf-mptb for growth promotion were between 8 and 128 pM. There was a decrease in the stimulation of growth at the 512-pM concentration. Similarly, the Rpf-tb significantly shortened the lag phase and enhanced the growth of the quiescent *M. tuberculosis* bacteria in a dose-dependent manner (Fig. 3b). The optimal concentrations of Rpf-tb for growth promotion were between 32 and 128 pM. In contrast to the



**Figure 3** Effect of Rpfs on the growth of mycobacteria. Mycobacteria were harvested by centrifugation, washed once, and resuspended in Sauton medium.<sup>18</sup> About  $10^2$  cells/ml were inoculated into Sauton medium in the presence or absence of Rpf and the OD<sub>600</sub> of the cultures were measured daily. Panel A: Effect of Rpf-mptb (■: 0, ▲: 8, ▼: 32, ◆: 128, or ○: 512 pM) on the growth of 2-month-old *M. avium* subsp. *paratuberculosis*, Panel B: Effect of Rpf-tb (■: 0, ▲: 8, ▼: 32, ◆: 128, or ○: 512 pM) on the growth of 1-month-old *M. tuberculosis* cells.

**Table 1** Effect of Rpf on the growth of *M. tuberculosis* H37Rv bacteria.

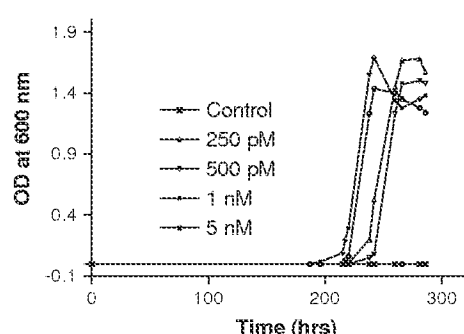
	Days	No Rpf (CFU/ml)	+ Rpf-tb (CFU/ml)	+ Rpf-mptb (CFU/ml)	+ Rpf-tb + Rpf-mptb (CFU/ml)
1-month-old culture	0	$1.2 \times 10^4$	$1.2 \times 10^4$	$1.2 \times 10^4$	$1.2 \times 10^4$
	6	$4.9 \times 10^4$	$5.2 \times 10^5$	$6.9 \times 10^5$	$6.4 \times 10^5$
	10	$3.1 \times 10^5$	$2.4 \times 10^7$	$3.9 \times 10^7$	$4.1 \times 10^7$
4-month-old culture	0	$3.7 \times 10^2$	$3.7 \times 10^2$	$3.7 \times 10^2$	$3.7 \times 10^2$
	6	$1.3 \times 10^3$	$1.8 \times 10^4$	$2.4 \times 10^4$	$1.7 \times 10^4$
	10	$6.5 \times 10^3$	$7.4 \times 10^5$	$8.3 \times 10^5$	$5.9 \times 10^5$

Rpf: Resuscitation promoting factor; CFU: colony-forming unit

Rpf-mptb results, there was not a decrease in the stimulation of growth at the 512-pM concentration but rather it leveled off.

To measure the effect on cell growth at earlier time points than possible with optical density measurements, *M. tuberculosis* H37Rv bacteria were harvested from a 1-month-old culture and used to inoculate Sauton media containing 128 pM Rpf-tb, 128 pM Rpf-mptb, 128 pM Rpf-tb and 128 pM Rpf-mptb, or no Rpf. The number of CFUs was determined at 0, 6, and 10 days post-inoculation (Table 1). At 6 days post-inoculation, 10- to 14-fold more CFUs were recovered from the cultures containing either of the Rpfs. At 10 days post-inoculation, 76- to 132-fold more CFUs were recovered from the cultures containing either of the Rpfs. The recovery of CFUs from the cultures containing the mixture of Rpfs was not significantly different than the recovery from the cultures with the individual Rpfs. Similar results were obtained when bacilli from a 4-month-old culture of *M. tuberculosis* H37Rv were used (Table 1).

Rpf activity can also be measured using washed *M. smegmatis* bacteria because when washed *M. smegmatis* cells are used to inoculate Sauton medium, there is no significant increase in CFUs during a 10-day incubation without the addition of Rpf to the medium.<sup>11</sup> The use of a relatively poor medium and low inoculum is required to observe the effect: that is, washed *M. smegmatis* cells will grow well in the absence of Rpf if a large inoculum is used ( $>10^2$  cells/ml) or if a rich medium such as Middlebrook 7H9 is used (data not shown). As shown in Fig. 4, extensively washed *M. smegmatis* bacteria from an early stationary phase culture were used to inoculate Sauton media ( $<10^2$  bacteria/ml) with or without Rpf-tb and growth followed by OD600 measurements. No measurable growth was detected in the cultures without Rpf-tb. Growth of the washed *M. smegmatis* cells was strongly stimulated in a dose-dependent manner



**Figure 4** Rpf-dependent growth of *M. smegmatis* 607. Bacteria from early stationary phase were recovered, washed in Sauton medium<sup>18</sup> 5 times, and used to inoculate a 50-ml flask containing 20 ml Sauton media with or without Rpf. The cultures were incubated at 37°C and OD600 measured daily. ■: Control without Rpf; ▲: 250 pM; ▼: 500 pM; ◆: 1 nM; and ×: 5 nM Rpf-tb.

when Rpf-tb was added at concentrations from 250 pM to 5 nM.

### MPN assay

To determine if the Rpfs were acting to reduce the lag phase or to 'resuscitate' dormant cells, MPN assays were done to determine the number of viable bacteria recoverable from a culture.<sup>11-13</sup> In an MPN assay, bacteria are diluted to such an extent that portions containing only one or a few viable cells are used to inoculate a set of replicate cultures and the number of test cultures in which bacteria grow is used to calculate the number of viable cells in the original sample. To measure the resuscitation activity of the mycobacterial Rpfs, an MPN assay was done using a 3-month-old *M. tuberculosis* H37Rv bacterium (Table 2). As is typically observed for old cultures of tubercle bacilli, there are many more bacteria in the culture

**Table 2** Resuscitation activity of Rpf on a 3-month-old *M. tuberculosis* H37Rv culture.

	Cells/ml	Recovery (%)
Total cell count	$6.9 \times 10^7$	—
CFU counts	$1.5 \times 10^5$	0.2
Sauton—no Rpf	$1.1 \times 10^6$	1.4
Sauton + Rpf-tb	$9.2 \times 10^6$	13
Sauton + Rpf-mptb	$1.6 \times 10^7$	23

Rpf: Resuscitation promoting factor; CFU: colony-forming unit.

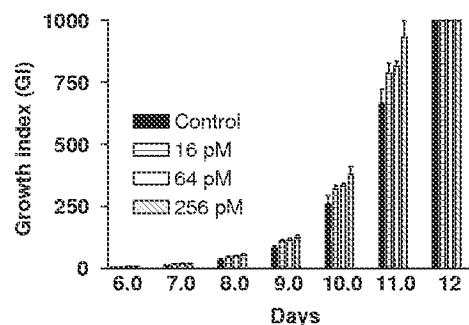
as determined by microscopy than there are bacteria capable of forming colonies when portions are plated directly onto solid media ( $6.9 \times 10^7$  bacteria vs  $1.5 \times 10^5$  CFU; Table 2). By MPN assay,  $1.1 \times 10^6$  viable cells (1.4% of the bacteria) could be recovered by inoculating Sauton medium. The addition of either the Rpf-tb (128 pM) or the Rpf-mptb (128 pM) resulted in the recovery of 8- to 15-fold more viable cells from the 3-month-old culture. The addition of either Rpf to the rich Middlebrook 7H9 medium did not enhance recovery (data not shown).

### BACTEC culture assay

The BACTEC 12B mycobacterial culture system was employed to further test the effect of Rpf-tb on the mycobacterial growth. The BACTEC 12B mycobacterial medium is an enriched Middlebrook 7H9 broth. In this system, the growth of mycobacteria is measured by the release of  $^{14}\text{CO}_2$  from  $^{14}\text{C}$ -labeled palmitic acid. The addition of Rpf-tb to BACTEC vials inoculated with bacteria from a 3-month-old culture of *M. tuberculosis* H37Rv only slightly increased the growth of the bacteria in a dose-dependent manner (Fig. 5), similar to the results in the studies with rich media described above.

### Discussion

Proteins with sequence homology to Rpf are widely distributed among member of GC-rich Gram-positive bacterial genera such as *Mycobacterium*, *Micrococcus*, *Streptomyces*, and *Corynebacterium*.<sup>10</sup> In database searches, 19 homologs of the *M. luteus* Rpf were identified in the genomes of several mycobacteria. For example, 5 Rpf homologs were found in the *M. tuberculosis* genome. The role



**Figure 5** Effect of Rpf-tb on the growth of *M. tuberculosis* in BACTEC cultures (Becton Dickinson and Company, Sparks, MD). Bacteria were inoculated into BACTEC<sup>®</sup> 12B culture vials in the presence of different concentrations of Rpf-tb, and the growth of the cultures was determined daily by the BACTEC-TB 460 system. The results represent the average of triplicate experiments.

of the individual Rpf proteins and reasons for redundancy are not yet known.

Initially, we chose 5 putative *rpf* genes (*Rv1009*, *Rv0867c*, *Rv2389c*, *Av27* and *Mptb*) for study. We chose these genes to reflect the variation in the Rpf homologs. *Rv1009* (362 residues) has a longer N-terminal sequences than does the *M. luteus* Rpf, *Rv0867c* has a longer C-terminal sequence, and *Rv2389c* has shorter N- and C-terminal sequences. *Av27* and *Mptb* were chosen as representatives from *M. avium* and *M. avium* subsp. *paratuberculosis*. Expression of *Rv0867c*, *Rv2389c* and *Av27* was poor in *E. coli* in our experiments. Similarly, Mukamolova et al.<sup>14</sup> reported obtaining only weak expression of *Rv0867c* and *Rv2389c* in their experiments.

In addition to the amino acid sequence homologies, three lines of evidence indicate that the products of the *Mptb* and *Rv1009* genes have resuscitation activity: the proteins can (i) shorten the lag time and promote the growth of bacteria cultured from a quiescent or stationary phase, (ii) increase the number of bacteria capable of growing that can be recovered from a quiescent or stationary phase culture, and (iii) support the growth of *M. smegmatis* bacteria that have been extensively washed and inoculated into Sauton medium. Our results with the *Rv1009* Rpf confirm the demonstration of the resuscitation activity of *Rv1009* by Mukamolova et al.<sup>15</sup> The ~10-fold increase in viable bacteria recovered from an *M. tuberculosis* 3-month-old culture is consistent with the effects seen in other systems with other resuscitation factors.<sup>10–12,14</sup>

The rapid and sensitive detection of mycobacteria in clinical specimens is important for the

laboratory confirmation of an infection. Because clinical specimens may contain a mixture of actively growing and quiescent mycobacteria, our initial interest in Rpfs arose from the possibility of improving the recovery of mycobacteria from clinical specimens. For example, studies aimed at recovering *M. avium* subsp. *paratuberculosis* bacteria from tissue specimens from Crohn's disease patients have had limited success and visible colonies take 6–18 months to appear,<sup>3,4</sup> if at all. Recovery of tubercle bacilli from sputum specimens can take as long as 8–10 weeks. In a minimal medium such as Sauton medium, both of the mycobacterial Rpfs could increase the recovery of mycobacteria by 10-fold or more. Unfortunately, very little, if any, improvement in recovery could be detected when rich media such as Middlebrook 7H9 or BACTEC media were used. This result may not be entirely unexpected because Middlebrook 7H9 media and the BACTEC media were developed to optimize the recovery of tubercle bacilli from clinical specimens.<sup>20–23</sup> Further studies are needed to determine if the mycobacterial Rpfs can improve the recovery of *M. tuberculosis* or *M. avium* subsp. *paratuberculosis* bacteria from clinical samples.

## Acknowledgements

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## References

- Centers for Disease Control and Prevention. Reported cases of tuberculosis in the United States, 2000. CDC, Division of Tuberculosis Elimination, Atlanta, GA. 2001.
- World Health Organization report 2002, Global tuberculosis control: surveillance, planning, financing. WHO report 2002, Geneva, Switzerland. WHO/CDS/TB/2002.295.
- Chiodini RJ, Van Kruiningen HJ, Merkkal RS. Ruminant paratuberculosis (Johne's disease): the current status and future prospects. *Cornell Vet* 1984;**74**:218–62.
- Collins MT, Lisby G, Moser G, Chicks D, Christensen S, Reichelderfer M, Hoiby N, Harms BA, Thomsen OO, Skibsted U, Binder V. Results of multiple diagnostic tests for *Mycobacterium avium* subsp. *paratuberculosis* in patients with inflammatory bowel disease and in controls. *J Clin Microbiol* 2000;**38**:4373–81.
- McFadden JJ, Butcher PD, Chiodini R, Hermon-Taylor J. Crohn's disease-isolated mycobacteria are identical to *Mycobacterium paratuberculosis*, as determined by DNA probes that distinguish between mycobacterial species. *J Clin Microbiol* 1987;**25**:796–801.
- Kaprelyants AS, Kell DB. Dormancy in stationary-phase cultures of *Micrococcus luteus*: flow cytometric analysis of starvation and resuscitation. *Appl Environ Microbiol* 1993;**59**:3187–96.
- Kaprelyants A, Mukamolova GV, Davey HM, Kell DB. Quantitative analysis of the physiological heterogeneity within starved cultured of *Micrococcus luteus* by flow cytometry and cell sorting. *Appl Environ Microbiol* 1996;**62**:1311–6.
- Votyakova TV, Kaprelyants AS, Kell DB. Influence of viable cells on resuscitation of dormant cells in *Micrococcus luteus* cultures held in an extended stationary phase: the population effect. *Appl Environ Microbiol* 1994;**60**:3284–91.
- Kaprelyants A, Mukamolova G V, Kell DB. Estimation of dormant *Micrococcus luteus* cells by penicillin lysis and by resuscitation in cell-free spent medium medium at high dilution. *FEMS Microbiol Lett* 1994;**115**:347–52.
- Mukamolova GV, Kaprelyants AS, Young DI, Young M, Kell DB. A bacterial cytokine. *Proc Natl Acad Sci USA* 1998;**95**:8916–21.
- Mukamolova GV, Kormer SS, Kell DB, Kaprelyants AS. Stimulation of the multiplication of *Micrococcus luteus* by an autocrine growth factor. *Arch Microbiol* 1999;**172**:9–14.
- Shleeva MO, Bagramyan K, Telkov MV, Mukamolova GV, Young M, Kell DB, Kaprelyants AS. Formation and resuscitation of "non culturable" cells of *Rhodococcus rhodochrous* and *Mycobacterium tuberculosis* in prolonged station phase. *Microbiology* 2002;**148**:1581–91.
- Biketov S, Mukamolova GV, Potapov V, Gilenkov E, Vostroknutov G, Kell DB, Young M, Kaprelyants AS. Culturability of *Mycobacterium tuberculosis* cells isolated from murine macrophages: a bacterial growth factor promotes recovery. *FEMS Immunol Med Microbiol* 2000;**29**:233–40.
- Mukamolova G, Turapov OA, Young DI, Kaprelyants AS, Kell DB, Young M. A family of autocrine growth factors in *Mycobacterium tuberculosis*. *Mol Microbiol* 2002;**46**:623–35.
- Sun Z, Zhang Y. Spent culture supernatant of *Mycobacterium tuberculosis* H37Ra improves viability of aged cultures of this strain and allows small inocula to initiate growth. *J Bacteriol* 1999;**181**:7626–8.
- Zhang Y, Yang Y, Woods A, Cotter R J, Sun Z. Resuscitation of dormant *Mycobacterium tuberculosis* by phospholipids or specific peptides. *Biochem Biophys Res Commun* 2001;**8**:542–7.
- Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning: a laboratory manual*, 2nd ed.. Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1989.
- Allen BW. Mycobacteria: general culture methodology and safety considerations. In: Parish T, Stoker NG, editors. *Mycobacteria protocols, methods in molecular biology*, vol. 101. Totowa, NJ: Humana Press, 1998. p. 10.
- Meynell GG, Meynell E. Theory and practice in experimental bacteriology. Cambridge, Cambridge University Press, 1970. p. 219–36.
- Anargyros PD, Astill DSJ, Sim IS. Comparison of improved BACTEC and Lowenstein-Jensen medium for culture of mycobacteria from clinical specimens. *J Clin Microbiol* 1990;**28**:1288–91.
- Kirihara JM, Hillier SL, Coyle MB. Improved detection times for *Mycobacterium avium* complex and *Mycobacterial*



- tuberculosis* with the BACTEC radiometric system. *J Clin Microbiol* 1985;**22**:841–5.
22. Park CH, Hixon DL, Ferguson CB, Hall SL, Risheim CC, Cook CB. Rapid recovery of mycobacteria from clinical specimens using automated radiometric technique. *Am J Clin Pathol* 1984;**81**:341–5.
23. Tokars JL, Rudnick JR, Kroc K, Manangan L, Pugliese G, Huebner RE, Chan J, Jarvis WR. US hospital mycobacteriology laboratories: status and comparison with state public health department laboratories. *J Clin Microbiol* 1996;**34**:680–5.